Fibronectin Fibrillogenesis Involves the Heparin II Binding Domain of Fibronectin*

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Fibronectin matrix assembly is thought to involve binding interactions between the amino-terminal I1–5 repeats and the first type III repeat (III1). Here we report that a third site, located within the III12–14 repeats of the carboxyl-terminal heparin II domain of fibronectin, is also involved in fibrillogenesis. Heparin II fragments inhibited fibril formation and binding of 125I-labeled fibronectin and/or 70-kDa fragments to the cell surface, deoxycholate-insoluble matrix, and adsorbed 160-kDa cell adhesion fragments of fibronectin. The inhibitory effects of heparin II fragments were as large or up to 20 times larger than those of a 44-kDa fibronectin fragment containing the III1 repeat. Under physiological conditions, amino-terminal fragments of fibronectin containing the I1–5 repeats interacted preferentially with proteolytically derived heparin II fragments and a recombinant III12–14 peptide both in solution and in solid phase, indicating that matrix assembly may involve direct interactions between I1–5 and III12–14 repeats. Interactions between the I1–5 repeats and 160-kDa fragments containing the III12–14 and III1 repeats could be inhibited by ≥90% by either an anti-III13–14 monoclonal antibody (mAb) (IST-2) or an anti-III1 mAb (9D2), suggesting that cooperative interactions between III12–14 and III1 repeats may also promote binding of the I1–5 repeats. Neither mAb IST-2 nor mAb 9D2, alone or in combination, inhibited binding of 125I-labeled 70-kDa fragments to cycloheximide-treated cells plated on the 160-kDa substrate, suggesting that additional I1–5 binding sites, independent of the III1 and III12–14 repeats, may be involved in fibrillogenesis.

Fibronectin is required for normal growth and development (1) and plays an important role in regulating cell attachment and movement, wound healing, and tumorigenesis (for review, see Refs. 2 and 3). It is a 500-kDa disulfide-bonded dimer consisting of similar subunits and is found as a soluble glycoprotein in blood and other body fluids and as an insoluble fibrous matrix component in tissues. Each subunit of fibronectin consists of three different types of repeating sequences, called types I, II, and III, which are arranged into discrete structural and functional modules.

Assembly of dimeric fibronectin into the extracellular matrix involves multiple consecutive binding interactions with integrin receptors, with itself, and with matrix components such as type I collagen (for review, see Refs. 4–6). Although the α5β1 integrin appears to be the primary fibronectin receptor involved in matrix assembly (7–12), at least two other integrins, α1β1 and α6β4, can also support fibronectin fibrillogenesis (13, 14). High affinity binding interactions between these integrins and the RGD site in the 10th type III repeat (III10) of fibronectin are thought to promote fibrillogenesis by exposing appropriate self-assembly sites in fibronectin. Such sites may become exposed through local integrin-induced conformational changes in III10 repeats (15) or through integrin-mediated stretching (reversible unfolding) of one or a whole array of type III repeats in fibronectin in response to cell movements (13, 16).

Self-assembly of fibronectin dimers into fibrils is currently thought to involve primarily interactions between the first five I1–5 repeats (I1–5) and the first type III (III1) repeat (17, 18). The I1–5 repeats are critical for matrix assembly, i.e. peptides including these repeats block assembly of fibronectin into fibrils, and fibronectin dimers lacking these repeats will not be incorporated into fibrils (19–22). The III1 repeats are also important for fibril formation, and either anti-III1 monoclonal antibodies or peptides derived from III1 repeats can block assembly of fibronectin into matrix (23, 24). The mechanism, however, by which I1–5-III1 interactions affect matrix assembly remains controversial. For example, Hocking et al. (25) have shown that III1 repeats will interact not only with I1–5 repeats but also with heat-denatured III10 repeats. They have proposed that the latter interaction activates the III1 repeat thereby allowing it to function as a receptor for the amino termini of a second fibronectin dimer. In contrast, Sechler et al. (26) have shown that fibronectin dimers lacking III1–7 repeats are readily polymerized into fibrils. In fact, the mutated dimers are rendered deoxycholate-insoluble more rapidly than intact dimers, suggesting that III1–7 repeats do not promote, but rather inhibit matrix assembly by keeping fibronectin in a compact form through intramolecular interactions with I1–5 repeats.

Earlier studies by Homandberg and Erickson (27) had indicated that another complementary binding site of the I1–5 repeats is located within the III12–14 repeats of the carboxyl-terminal heparin II binding domain of fibronectin, but the functional significance of this site was never investigated. Here we report that the heparin II domain plays an important role in matrix assembly, and we demonstrate that peptides containing the III12–14 repeats specifically block binding of fibronectin and its 70-kDa amino-terminal fragments to cells and their matrix. We also present evidence indicating that the inhibitory effects of the heparin II domain may involve direct interactions between the I1–5 and the III12–14 repeats and that binding of the I1–5 repeats may depend on cooperative interactions between the III12–14 and III1 repeats. Finally, the data presented here confirm our earlier report (12) and show that the amino terminus participates in additional binding interactions that are independent of either the III1 or the III12–14 repeats.
FiguRe 1. Schematic diagram of fibronectin indicating the fibronectin fragments and antibodies used. The diagram represents a monomer of plasma fibronectin consisting of type I (rectangles), type II (ovals), type III (numbered squares) repeats, and the variable connecting sequence (CS). The two main heparin-binding domains (Hep I, Hep II) are also indicated. The location of the epitopes recognized by the monoclonal antibodies used in this study are indicated above. The location of the proteolytic fragments used in this study are indicated below in concert with their molecular mass. The proteolytically derived Hep II-binding fragments consisting of five peptides and their weighed average molecular mass (50 kDa) are indicated by a solid line. Some of these fragments may extend further as indicated by the dotted line.

MATERIALS AND METHODS

Cell Culture and Binding Assays—Neonatal human skin fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Binding assays in confluent cultures of matrix-deprived cells were performed as described before (12). Briefly, cells were harvested with trypsin and EDTA, washed in the presence of soybean trypsin inhibitor, and kept in suspension for 1 h at 21 °C in binding buffer (Dulbecco’s modified Eagle’s medium containing 25 μg/ml cycloheximide, 2 mg/ml BSA, 100 units/ml penicillin G, 5 μM streptomycin sulfate, and 25 mM Hepes, pH 7.4). Cells were then pelleted in 96-well polystyrene microtiter wells precoated with 40–120 μg/ml of the 160-kDa or 5 μg/ml of the 75-kDa cell adhesion fragments of fibronectin. These coating concentrations had been shown previously to be optimal for cell spreading and binding of 70-kDa fragments (12). Plates were blocked overnight with 2 mg/ml heat-denatured BSA (5 min at 80 °C). In some experiments, antibodies were added to the cell suspension just before plating. After 3 h at 37 °C, cells were incubated with 125I-labeled fibronectin (1 × 10^6 cpm/ml) or 70-kDa fragments (10^7 cpm/pmol) or 70-kDa fragments (1–3 × 10^6 cpm/ml) for 1 or 2 h at 37 °C, and wells were washed, separated, and counted.

Binding interactions between ligand and substrate which occur in the absence of cells were also measured in microtiter wells as described previously (12). For some of these experiments (see Fig. 6 and 7), the pH of the binding buffer was adjusted to 6.6 with HCl. Nonspecific binding was measured either in wells that had been coated only with the blocking agent (2 mg/ml heat-denatured BSA) or in the presence of a 100-fold excess of unlabeled ligand. Nonspecific binding measured in the presence of excess competitor represented 10–20% and 20–50% of the total binding in the presence and absence of cells, respectively, and was independent of the addition of any of the antibodies.

Matrix Assembly Assay—Cells were plated at confluence and maintained in the presence of 10% fetal bovine serum for 3 days. Cells plated in 35-mm dishes were then incubated with 0.1 mM 125I-labeled 70-kDa fragments (2 × 10^6 cpm/ml) in 1 ml of binding buffer, pH 7.4, for 2 h at 37 °C with gentle rocking. Cells plated in 80-mm dishes were incubated with 0.75 mM 125I-labeled 70-kDa fragments (2 × 10^6 cpm/ml) in 0.25 ml of binding buffer, pH 7.4, for 2 h at 37 °C with gentle rocking. The cells were washed three times with binding buffer and lysed at 4 °C with 1% deoxycholate in hypotonic buffer (5 mM Tris-HCl, pH 8.3, containing 1 mM NaCl, 2 μg/ml leupeptin, and 2 μg/ml aprotinin) and scraped from the dishes. The deoxycholate-soluble cellular fraction (pool I) and insoluble matrix fraction (pool II) were separated by centrifugation at 35,000 × g as described (28). The deoxycholate-insoluble material was washed with 1% deoxycholate in hypotonic buffer and recentrifuged. All operations were done at 4 °C.

Isolation of Adherent Matrices—Adherent matrices were prepared from fibroblast cultures that had been plated at confluence in 60-mm dishes and maintained in the presence of 10% fetal bovine serum for 3 days. Cells were lysed with 1% deoxycholate in hypotonic buffer for 5 min (28). The lysates in the plates were treated with DNase I (50 units/ml; Sigma), MgCl2, and CaCl2 (5 mM each) in hypotonic buffer for 15 min, and the plates were rinsed with hypotonic buffer followed by binding buffer. The fibrous matrices, prepared in this way at 21 °C, remained firmly attached to the dishes in subsequent binding assays. Binding assays using isolated matrices were done as described above for intact cell layers.

1 The abbreviations used are: BSA, bovine serum albumin; PBS, phosphate-buffered saline; BS5, bis(sulfosuccinimidyl) suberate; mAb, monoclonal antibody; GST, glutathione S-transferase.
Heparin II Domain of Fibronectin Inhibits Fibrillogenesis

Dr. Luciano Zardi, Instituto Nationale per La Ricerca Sul Cancro. The antibodies were concentrated by precipitation with 30% ammonium sulfate and/or solvent extraction with flakes of polyethylene glycol followed by dialysis against 20 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl. IgG concentrations were determined by using a mouse IgG kit (Boehringer Mannheim).

Preparation of Recombinant Type III12–14 Peptide—Human full-length fibronectin cDNA, pFH100 (33), was used as a template for polymerase chain reaction amplification (34) of a DNA sequence (bases 5072–5923) encoding the III12–14 repeats of fibronectin (amino acid Glu1067 through Pro1070). The DNA was generously provided by Dr. Deane Mosher (who received it from Dr. Jean Paul Thiery, CNRS URA, Paris). The bases are numbered starting with the G before the first codon of the first amino acid of the mature protein (GenBank™ accession no. X02761), and the amino acids are numbered starting with the pyroglutamic acid (35). This region spans the entire length of the III12–14 repeats and also includes 3 amino acids upstream of the III12 repeat (QST) and also includes 3 amino acids downstream of the III14 repeat (DELPLQVLTP). The sense primer, 5'-GAATTC-CAGTCGACAGCTATTCCTGT, generated an EcoRI site (in boldface), whereas the antisense primer, 5'-CTCGAGATTATCGAGGTTACGATTG, generated an XhoI site (in boldface) and also introduced a stop codon (underlined). The polymerase chain reaction-amplified DNA was purified using the Wizard™ PCR Prep DNA Purification System (Promega, Madison, WI) and subsequently ligated into the pGEM-T vector (Promega) according to the manufacturer’s instructions. The pGEM-T vector containing the insert was transfected into electroporation (ElectroCell Manipulator 600 BTX Electroporation System) into JM109 bacteria. The pGEM-T vectors containing the appropriate sized insert were digested with EcoRI and XhoI, and the cDNA fragment encoding the III12–14 repeats was gel purified using the Wizard™ PCR Prep DNA Purification System. This purified DNA fragment was then cloned in-frame in the bacterial expression vector pGEX-4T1 (Pharmacia) and electroporated as described above. Expression of the glutathione S-transferase fusion peptide (GST-III12–14 repeats) was confirmed using Western blot analysis with mAb IST-2.

The GST-III12–14 fusion peptide was purified using modifications of the procedure described previously (36). The modifications included adding 1 mM Pefabloc®SC (Boehringer Mannheim) and lysing the cells with a French press followed by sonication (three cycles of 10 s each). The fusion peptide was adsorbed to glutathione-Sepharose 6B beads (Pharmacia) and digested overnight at 4 °C with thrombin (10 NIH units/500 ml of original culture). The beads were removed by centrifugation at 250 x g for 5 min at 4 °C. The supernatant containing the cleaved fusion peptide was incubated with p-aminobenzamidine Sepharose 6B beads (Sigma) for 20 min at 4 °C to remove thrombin. The beads were removed by centrifugation at 250 x g for 5 min, and the recombinant III12–14 peptide in the final supernatant was concentrated on flakes of polyethylene glycol and dialyzed into PBS. SDS-polyacrylamide gel electrophoresis and Western blots showed that the recombinant III12–14 peptide migrated as a doublet (~29-kDa) and was recognized by the mAb IST-2 (data not shown). Western blot analysis using a goat anti-GST serum (gift from Dr. James Tracy, University of Wisconsin, Madison) showed that the recombinant peptide did not contain any uncleaved fusion peptide or GST (data not shown).

Immunofluorescence Microscopy—Teflon® coated 12-well slides (Polysciences, Warrington, PA) were precoated for 2 h at 21 °C with 20 µg/ml of the 160-kDa fragment of fibronectin in Hanks’ balanced salt solution. Fibroblasts suspended in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, 100 units/ml penicillin G, and 5 µM streptomycin sulfate were plated at a density of 3 x 104 cells/well and incubated at 37 °C to allow the cells to attach. After 1 h, the medium was replaced with serum-free medium (Dulbecco’s modified Eagle’s medium, 2 mg/ml BSA, 25 µg/ml cycloheximide, 100 units/ml penicillin G, and 5 µM streptomycin sulfate), and the cells were allowed to spread for 3 h at 37 °C. The medium was removed again and replaced with fresh serum-free medium containing 1 µg/ml human plasma fibronectin. In some wells, either the 70-kDa (93 µg/ml) or heparin II-containing fragments (290 µg/ml) were added as inhibitors. Fibroblasts were kept overnight at 37 °C, washed with Hanks’ balanced salt solution, and fixed with 4% paraformaldehyde, 0.1 M sodium phosphate buffer at pH 7.4 for 30 min at 21 °C. Fibronectin fibrils were labeled with a polyclonal anti-fibronectin serum as described previously (37) and viewed by epifluorescence with a Nikon Optiphot microscope. Images were digitized using a Photometrics Image Point® CCD camera and Image Pro Plus® program.

RESULTS

Heparin II-containing Fragments of Fibronectin Inhibit Assembly of Fibronectin into Matrix—Earlier studies by Homandberg and Erickson (27) had indicated that the heparin II binding domain of fibronectin may contain an amino-terminal binding site. Because an amino-terminal binding site in the III1 repeat of fibronectin had been shown to play an important role in the assembly of fibronectin into fibrils (17, 18, 25, 26), we wanted to know whether the heparin II binding domain of fibronectin also participates in fibrillogenesis. To examine this question, cycloheximide-treated fibroblasts capable of assembling exogenous plasma fibronectin into fibrils (38) were tested for their ability to assemble plasma fibronectin into fibrils in the presence, or absence, of proteolytically derived fragments of fibronectin. In the absence of any fibronectin fragments, numerous fibronectin fibrils were observed in these cultures (arrowheads, Fig. 2a). If, however, fragments of fibronectin containing the heparin II domain were added, fibril formation was inhibited completely (Fig. 2c), indicating that the heparin II domain may indeed play a role in fibrillogenesis. The 70-kDa amino-terminal fragment of fibronectin, as expected (19, 39), also blocked incorporation of exogenous fibronectin into matrices (Fig. 2b).

The inhibitory effect of the heparin II domain on fibrillogenesis was confirmed biochemically using the matrix assembly assay described by McKeown-Longo and Mosher (19, 28). In this assay, incorporation of 125I-fibronectin into the deoxycholate-insoluble matrix (pool II) was inhibited with increasing concentrations of unlabeled 70-kDa fragments (Fig. 3). The IC50 of the inhibition was 1.0 µM. At 10-fold higher concentrations, fragments containing the III12–14 repeats were equally effective inhibitors (IC50 = 10 µM). The 44-kDa fragment containing the III14 repeats was also inhibitory at these higher concentrations, however it never blocked incorporation of 125I-fibronectin by more than 40%. In contrast, 75-kDa fragments containing the III11–10 repeats or BSA had little (20%) or no inhibitory effects.

FIG. 2. Inhibition of fibronectin fibrillogenesis in the presence of the 70-kDa or heparin II-containing fragments. Cycloheximide-treated fibroblasts plated on the 160-kDa fragment of fibronectin were incubated overnight with plasma fibronectin (panel a), plasma fibronectin and the 70-kDa amino-terminal fragment (panel b), or plasma fibronectin and heparin II-containing fragments (panel c) as described under “Materials and Methods.” At the concentrations used, 70-kDa (1.5 µM) and heparin II fragments (4.4 µM) inhibited binding of 125I-fibronectin by 50 and 40%, respectively (see Fig. 4A). A phase image of the cell layer is shown in panel d. All cultures were fixed and labeled with an anti-fibronectin serum followed by an anti-rabbit IgG conjugated to rhodamine as described under “Materials and Methods.” Fibronectin fibrils (arrowheads) can be found only in cultures incubated with plasma fibronectin alone. Cultures incubated with either fibronectin fragment did not form fibrils. Bar = 50 µm.
inhibited binding of 125I-fibronectin to the cell surface in confluent cell layers of matrix-deprived cycloheximide-treated fibroblasts plated on the 75-kDa cell adhesion fragment of fibronectin (12). As shown in Fig. 4A, the 70-kDa fragment was again the most effective inhibitor (IC50 = 0.1 μM) followed by the heparin II (IC50 = 3 μM) and the 44-kDa (IC50 = 20 μM) fragments. The 75-kDa fragment and BSA had little or no inhibitory effect. Thus, under physiological conditions, III1–5-containing fragments inhibited binding of 125I-fibronectin to cell layers at least 10 times more efficiently than either the III1–4- or III5–10-containing fragments. These studies clearly establish that besides the amino-terminal I1–5 repeats, the type III repeats contained in the carboxy-terminal heparin II-binding fragments are the most effective inhibitor of fibronectin matrix assembly and that they are capable of inhibiting the initial binding of fibronectin to the cell surface as well as its subsequent incorporation into fibrils.

**Heparin II-containing Fragments Inhibit Binding of 125I-Labeled 70-kDa Fragments to Cells, 160-kDa Fibronectin Fragments, and Matrix Fibris**—Because the assembly of fibronectin into fibrils is thought to be mediated by the amino-terminal I1–5 repeats (19, 20), we also examined whether the heparin II-binding fragments could interfere directly with binding of 125I-labeled 70-kDa fragments to cells. As shown in Fig. 4B, unlabelled 70-kDa (IC50 = 0.006 μM), heparin II (IC50 = 2 μM), and 44-kDa (IC50 = 20 μM) fragments inhibited binding of 125I-labeled 70-kDa fragments to cells in almost exactly the same manner as they inhibited binding of 125I-fibronectin (see Fig. 4A). Thus, all of the binding interactions of fibronectin in cell layers which can be specifically inhibited by these fibronectin fragments appear to be I1–5-dependent binding interactions. In control experiments, high concentrations of BSA elevated the binding of 125I-labeled 70-kDa fragments slightly.

To test whether the inhibitory effects of heparin II fragments involve direct fibronectin-fibronectin binding interactions independent of cells, we measured the ability of these fragments to inhibit direct binding of 125I-labeled 70-kDa fragments to 160-kDa cell adhesion fragments of fibronectin adsorbed to plastic wells (12). This reaction was specifically inhibited by unlabeled 70-kDa (IC50 = 0.02 μM), heparin II (IC50 = 0.1 μM), and 44-kDa fragments (IC50 = 2 μM), whereas the 75- and 160-kDa cell adhesion fragments had the same nonspecific effect as high concentrations of BSA (Fig. 5). Both the heparin II fragments and the 44-kDa fragments thus inhibited binding of the 125I-labeled 70-kDa ligand to the 160-kDa substrate 10 times more...
Deoxynucleotide soluble (pool I) and insoluble fractions (pool II) and isolated fibronectin matrices were prepared as described under "Materials and Methods." Percent inhibition of specific binding refers to the percent inhibition by the heparin II containing fragments (hep II) relative to the percent inhibition by the 70-kDa fragment (0.8 μM). All data represent the means of four measurements ± S.E. The differences between controls and dishes treated with the inhibitor were significant at the 95% confidence level in each of the seven experiments (t test; comparison of means).

| Fraction and inhibitor | 125I-70-kDa fragments bound |
|------------------------|-----------------------------|
|                        | Control | Inhibitor | Inhibition of total binding | Inhibition of specific binding |
| μM | fmol/dish | % | % |
| Pool I | Hep II (4.0) | 4.42 ± 0.19 | 3.74 ± 0.19 | 15 | 42 |
| 70 kDa (0.8) | 4.27 ± 0.15 | 2.72 ± 0.12 | 36 | 70 |
| Pool II | Hep II (4.0) | 3.41 ± 0.14 | 2.57 ± 0.15 | 25 | 69 |
| 70 kDa (0.8) | 3.30 ± 0.18 | 2.10 ± 0.13 | 36 | 70 |
| Isolated matrix | Hep II (4.0) | 1.99 ± 0.19 | 1.41 ± 0.06 | 29 | 88 |
| 70 kDa (0.8) | 2.27 ± 0.25 | 1.53 ± 0.07 | 33 | 70 |
| 70 kDa (4.0) | 2.63 ± 0.26 | 1.17 ± 0.14 | 56 | 66 |

Fig. 5. Inhibition of the binding of 125I-labeled 70-kDa fragments to adsorbed 160-kDa substrate by fibronectin fragments containing the III12-14 or the III1 repeats. Microtiter wells, which had absorbed 2.9 pmol of 160-kDa fragments, were labeled with 2.8 ng 125I-labeled 70-kDa fragments (5 × 10^7 cpm/well) in 60 μl of binding buffer, pH 7.4, for 2 h at 37 °C in the absence or presence of unlabeled 70-kDa fragments (•), fragments containing the III12-14 repeats (○), or 44-kDa fragments containing the III1 repeat (▲) at the indicated concentrations. Nonspecific effects caused by increasing the protein concentration were measured by supplementing the binding buffer with additional BSA (△). All data represent means of triplicate measurements (bars indicate the S.E.) normalized to the binding of 125I-labeled 70-kDa fragments seen in the absence of any additions (0.90 ± 0.07 fmol/well; n = 9). Curves are third order regression lines.

The incorporation of 125I-labeled 70-kDa fragments into pool I, pool II, and isolated matrices was equally significantly (p > 0.05) inhibited by the addition of unlabeled heparin II-binding fragments. At the concentration chosen (4 μM), heparin II fragments inhibited 15, 25, and 29% of the total binding or 42, 69, and 88% of the specific binding of 125I-labeled 70-kDa fragments into pool I, pool II, and isolated matrices, respectively. We conclude, therefore, that the amino terminus of fibronectin can interact directly with the matrix in cell layers and that the heparin II-binding fragment can interfere with such binding significantly. Presumably, the ability of the heparin II fragments to inhibit the binding interaction between the 70-kDa ligand and the adsorbed 160-kDa substrate (Fig. 5) is physiologically significant in that it reflects this aspect of fibronectin matrix assembly.

**Direct Binding Interactions between I1-5 and III12-14 Repeats**—To determine whether direct interactions between I1-5 and III12-14 repeats could account for the inhibitory effects of heparin II-binding fragments in fibrillogenesis, 70-kDa fragments adsorbed to plastic wells were tested for their ability to bind proteolytically derived and recombinant peptides containing the III12-14 repeats. As shown in Fig. 6, 125I-labeled heparin II-containing peptides bound to adsorbed 70-kDa fragments in a concentration-dependent manner. On a molar basis, the recombinant peptide was bound three to four times more efficiently than the proteolytic fragments. Binding of radioiodinated recombinant and proteolytic fragments was inhibited by ~50% in the presence of a 300–400-fold molar excess of unlabeled heparin II-binding fragments. No further inhibition could be achieved by increasing the concentration of the competitor (data not shown). Presumably, this was because of the self-association of the heparin II-binding fragments at the higher concentrations (27). Binding interactions between heparin II-binding fragments and the 70-kDa fragments involve the amino-terminal I1-5 repeats. As shown in Fig. 7, binding of 125I-labeled amino-terminal fragments to increasing concentrations of adsorbed heparin II fragments is associated exclusively with the 29-kDa fragments containing the I1-5 repeats and not with the 40-kDa gelatin binding domain. In the experiments reported in Figs. 6 and 7, binding reactions were carried out at pH 6.6 because binding was considerably enhanced at this pH compared with the binding seen at pH 7.4 (data not shown).

Adsorbed 70-kDa fragments clearly bound recombinant and proteolytically derived III12-14-containing peptides in preference to other fibronectin fragments such as the 44-, 75-, or 40-kDa fragments (Fig. 8A). Such preferential binding was not merely an artifact caused by denaturation of the adsorbed 70-kDa fragment, but could also be demonstrated in solution. As shown in Fig. 8B, soluble biotinylated 70-kDa fragments bound the recombinant and the proteolytically derived III12-14-containing peptides two or three times more efficiently than

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**Heparin II Domain of Fibronectin Inhibits Fibrillogenesis**

2605

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**TABLE I**

Inhibition of amino-terminal binding to cell layers and isolated matrices by fragments containing the III12-14 repeats

| Fraction and inhibitor | 125I-70-kDa fragments bound |
|------------------------|-----------------------------|
|                        | Control | Inhibitor | Inhibition of total binding | Inhibition of specific binding |
| μM | fmol/dish | % | % |
| Pool I | Hep II (4.0) | 4.42 ± 0.19 | 3.74 ± 0.19 | 15 | 42 |
| 70 kDa (0.8) | 4.27 ± 0.15 | 2.72 ± 0.12 | 36 | 70 |
| Pool II | Hep II (4.0) | 3.41 ± 0.14 | 2.57 ± 0.15 | 25 | 69 |
| 70 kDa (0.8) | 3.30 ± 0.18 | 2.10 ± 0.13 | 36 | 70 |
| Isolated matrix | Hep II (4.0) | 1.99 ± 0.19 | 1.41 ± 0.06 | 29 | 88 |
| 70 kDa (0.8) | 2.27 ± 0.25 | 1.53 ± 0.07 | 33 | 70 |
| 70 kDa (4.0) | 2.63 ± 0.26 | 1.17 ± 0.14 | 56 | 66 |
they bound the 44-, 75-, or 40-kDa fragments, both in the absence and the presence of a homobifunctional cross-linker (BS3). These studies leave little doubt that direct I1–5–III12–14 interactions contribute to the inhibitory effects of the heparin II-binding fragments. The modest preferential binding interactions between the heparin II and 70-kDa fragments seen in solution, however, suggest that direct binding interactions cannot readily account for the fact that the inhibitory effect of the heparin II fragments may be 20 times larger than those of the 44-kDa fragments (see Fig. 5). Thus, the III12–14 repeats may also affect binding interactions of the I1–5 repeats by other mechanisms.

mAbs IST-2 and 9D2 Inhibit Binding of Amino-terminal Fragments of Fibronectin to the 160-kDa Substrate but Not to Cells—As shown in Figs. 3–5, maximal inhibition of the binding of 125I-fibronectin and 125I-labeled 70-kDa fragments by either the heparin II or the 44-kDa fragments does not plateau and may not be limited at all (note a possible exception in Fig. 3, △). This suggests that the inhibitory effects of the III12–14 and III1 repeats may not be independent of each other. To examine this possibility, we compared the roles of the III12–14

FIG. 6. Binding of recombinant and proteolytically derived III12–14-containing peptides to adsorbed 70-kDa fragments. Microtiter wells that had been coated with the indicated amounts of the 70-kDa fragment were labeled for 4 h at pH 6.6 and 37 °C with 0.86 pmol/well 125I-labeled recombinant III12–14 peptide (2.7 × 10^5 cpm/well) (●) or 0.57 pmol/well 125I-heparin II-binding proteolytic fragments (4.6 × 10^5 cpm/well)(▲, △) in the absence (filled symbols) and in the presence of 240 pmol/well unlabeled 50-kDa fragments (open symbols). Data represent means of triplicate measurements. S.E. (data not shown) were all <0.8 fmol/well. Binding of the recombinant fragment was normalized to the input of the proteolytic fragment. Nonspecific binding to BSA-coated wells was subtracted. Curves are third order regression lines.

FIG. 7. Comparison of 29-, 40-, and 70-kDa amino-terminal fragments binding to adsorbed heparin II-binding fragments. 125I-Labeled 29-kDa (18.3 nM or 2.3 × 10^5 cpm/well) (●), 70-kDa fragments (11.9 nM or 5.3 × 10^5 cpm/well) (□), or 40-kDa gelatin-binding fragments (12.5 nM or 3.7 × 10^5 cpm/well) (○) were incubated at pH 6.6 for 4 h at 37 °C in microtiter wells coated with increasing concentrations of heparin II-binding fragments. The data have been normalized to a ligand input of 10 nM, and they represent the means of triplicate measurements. Bars indicate the S.E. Curves are third order regression lines.

FIG. 8. Binding interactions between biotinylated 70-kDa fragments and various other fragments of fibronectin. Binding reactions were done in a solid phase assay (panel A) or in solution (panel B). In panel A, the proteolytically derived heparin II fragment (7.4 × 10^5 cpm/pmol), the recombinant III12–14 peptide (3.3 × 10^5 cpm/pmol), the 44-kDa fragment containing the III1 repeat (4.8 × 10^5 cpm/pmol), the 75-kDa cell adhesion fragment (8.1 × 10^5 cpm/pmol), or the 40-kDa gelatin-binding fragment of fibronectin (8.2 × 10^5 cpm/pmol) was bound to biotinylated 70-kDa fragments (30 μg/ml) absorbed to microtiter wells. Binding was done for 4 h at 37 °C in PBS, pH 7.4, containing 2 mg/ml BSA. Binding to uncoated BSA-blocked wells was subtracted from the data. Nonbiotinylated 70-kDa fragments (adsorbed at 30 μg/ml) bound the various ligands in exactly the same way as the biotinylated substrate (data not shown). In panel B, soluble biotinylated 70-kDa fragments were incubated with the various 125I-fibronectin fragments as described under "Materials and Methods." Binding was measured in the presence (hatched bars) and the absence (open bars) of the cross-linker BS3. In both panels A and B, the data represent the means of triplicate measurements. Bars indicate the S.E.
the other (see below, Fig. 11, centra-}

cations. Thus, either of the antibodies could substitute for

smooth curve by 30–40% in the presence of either the mAb IST-2 (0.1–10

substrate (Fig. 11, shown), or when both antibodies were used together (Fig. 11;)

pendent manner by 

inhibits binding of 125I-labeled 70-kDa fragments in a concentration-de-

ations (Fig. 9; ). The difference is even more striking because at equal 

concentrations mAb C6F10 binds to the 160-kDa 

fragment at least 100-fold more efficiently than mAb IST-2 (data not shown). Nonimmune mouse IgG is noninhibitory in this system (Fig. 9; ). Apparently, mAb IST-2 interferes with binding interactions of the type I1–5 repeats because it inhibits binding of 125I-labeled 29-kDa fragments as efficiently as it inhibits binding of 125I-labeled 70-kDa fragments (data not shown). If mAb 9D2 was used, binding of the 70-kDa ligand to the 160-kDa substrate was also blocked in a concentration-de-

ependent manner by > 90% provided that the antibody concen-

trations were increased ≥ 10-fold over the effective IST-2 con-

centrations. Thus, either of the antibodies could substitute for the other (see below, Fig. 11, ).

When cycloheximide-treated cells were plated on the 160-

do-10 μg/ml) or the mAb 9D2 (data not shown). Binding to cell layers

was unaffected by the mAb C6F10 or nonimmune IgG (Fig. 10).

In all of these experiments, antibodies were present from the
time cells were plated to the end of the labeling period. This

procedure ensured that the antibodies had access to the 160-
do-10 cell/well) were labeled for 1 h with the 

125I-labeled 70-kDa fragment (2 × 105 cpm/well or 0.1 pmol/well). In the

same experiment, cell-independent binding of the 70-kDa fragment to the

160-kDa substrate was measured in the presence of mAb IST-2 as described in Fig. 3 ( ). All data are means of triplicate measurements of specific binding normalized to specific binding seen in the absence of 

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DISCUSSION

In this paper we demonstrate that the carboxyl-terminal heparin II binding domain of fibronectin is involved in the assembly of fibronectin into fibrils. Using binding assays and immunofluorescence microscopy, we show that proteolytic fragments of fibronectin containing the III12–14 repeats inhibit binding of fibronectin, or its amino terminus, to cell layers and assembly of fibronectin into fibrils. Until now, the III1 repeat was the only type III repeat known to inhibit fibronectin fibril formation (23, 24). Under physiological conditions, the heparin II-binding fragments inhibited binding of fibronectin and amio-
terminal 70-kDa fragments to cells ≥ 10 fold more effi-
ciently than a 44-kDa fragment containing the III1 repeat. We conclude therefore that assembly of fibronectin fibrils involves at least two separate and distinct type III repeats.

The sequences within the heparin II binding domain which inhibit fibril formation are unknown. These sequences, like the inhibitory sequences within the III1 repeat, appear to be cryptic in fibronectin because a 160-kDa fragment of fibronectin containing the III12–14 and III1 repeats did not inhibit either the incorporation of fibronectin into the extracellular matrix or the binding of 70-kDa fragments to absorbed 160-kDa fragments.

and III1 repeats in the binding of 125I-labeled 70-kDa fragments with the 160-kDa substrate using anti-III13–14 (IST-2) and anti-III1 (9D2) monoclonal antibodies.

As shown in Fig. 9, the mAb IST-2 inhibited binding of 70-kDa fragments in a concentration-dependent manner by at least 80% and in some experiments by > 90% (Fig. 9; ). In contrast, mAb C6F10, recognizing the III8–10 repeats, inhibited binding by no more than 20% over a wide range of concentrations (Fig. 9; ). The difference is even more striking because at equal IgG concentrations mAb C6F10 binds to the 160-kDa fragment at least 100-fold more efficiently than mAb IST-2 (data not shown). Nonimmune mouse IgG is noninhibitory in this system (Fig. 9; ). Apparently, mAb IST-2 interferes with binding interactions of the type I1–5 repeats because it inhibits binding of 125I-labeled 29-kDa fragments as efficiently as it inhibits binding of 125I-labeled 70-kDa fragments (data not shown). If mAb 9D2 was used, binding of the 70-kDa ligand to the 160-kDa substrate was also blocked in a concentration-de-

ependent manner by > 90% provided that the antibody concen-

trations were increased ≥ 10-fold over the effective IST-2 con-

centrations. Thus, either of the antibodies could substitute for the other (see below, Fig. 11, ).

When cycloheximide-treated cells were plated on the 160-

kDa substrate, binding of 125I-labeled 70-kDa fragments in the absence of the antibodies was equal to the binding to the substrate alone (e.g. in Fig. 10, specific binding was 0.5 fmol/ well in the presence as well as in the absence of cells), or it was enhanced up to 3-fold (12). Surprisingly, none of the binding of 125I-labeled 70-kDa fragments to these cell layers was inhibited by either the mAb IST-2 (Fig. 10), the mAb 9D2 (data not shown), or when both antibodies were used together (Fig. 11; ), even though both antibodies could inhibit binding to the substrate (Fig. 11, ). Instead, binding to cells was enhanced by 30–40% in the presence of either the mAb IST-2 (0.1–10 μg/ml) or the mAb 9D2 (data not shown). Binding to cell layers

was unaffected by the mAb C6F10 or nonimmune mouse IgG (Fig. 10).

In all of these experiments, antibodies were present from the
time cells were plated to the end of the labeling period. This

procedure ensured that the antibodies had access to the 160-
do-10 cell/well) were labeled for 1 h with the 

125I-labeled 70-kDa fragment (2 × 105 cpm/well or 0.1 pmol/well). In the

same experiment, cell-independent binding of the 70-kDa fragment to the

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This leaves the possibility that the III\textsubscript{12–14} repeats may also inhibited by the mAbs IST-2 and 9D2. Erickson (27) and show that I\textsubscript{1–5} repeats bind preferentially to terminus.

inhibitory because they can bind to the I\textsubscript{1–5} repeats in either monolayers (3) by any of the antibody treatments (not shown). After 3 h, the confluent monolayers (3 × 10\textsuperscript{4} cells/well) were labeled for 1 h with \textsuperscript{125}I-labeled 70-kDa fragments (2 × 10\textsuperscript{5} cpm/well or 0.1 pmol/well). Binding to substrate alone was measured at the same time. Data represent means of triplicate measurements of specific binding normalized to specific binding seen in the absence of antibodies (0.5 fmol/well in the presence or absence of cells). Bars represent the S.E.

The data presented in this paper suggest two alternative mechanisms by which the heparin II binding domain could inhibit the incorporation of fibronectin into fibrils. First, the III\textsubscript{12–14} repeats could bind directly to the I\textsubscript{1–5} repeats and inactivate the amino terminus. Second, the heparin-II binding fragments could block cooperative interactions between the III\textsubscript{12–14} and III\textsubscript{1} repeats which facilitate binding of the amino terminus.

Our studies confirm previous studies by Homandberg and Erickson (27) and show that I\textsubscript{1–5} repeats bind preferentially to the III\textsubscript{12–14} repeats, both in solid phase and in solution. This is consistent with the idea that direct I\textsubscript{1–5}-III\textsubscript{12–14} interactions may contribute to fibronectin fibrillogenesis. It is also consistent with the idea that heparin II-containing fragments are inhibitory because they can bind to the I\textsubscript{1–5} repeats in either fibronectin or the 70-kDa fragment and form a complex that is incapable of binding to cells or the 160-kDa cell adhesion substrate. It remains uncertain, however, to which extent direct I\textsubscript{1–5}-III\textsubscript{12–14} interactions can account for the inhibitory effects of the heparin II-binding fragment because compared with the 44-kDa III\textsubscript{1}-containing fragment, III\textsubscript{12–14}-containing fragments bound soluble 70-kDa fragments only two to three times more efficiently whereas they could inhibit binding of 70-kDa fragments to cells or to the 160-kDa substrate ≥ 10 times better. This leaves the possibility that the III\textsubscript{12–14} repeats may also facilitate binding of I\textsubscript{1–5} repeats by alternative mechanisms.

One possible alternative mechanism suggested by our finding is that the IST-2 as well as the 9D2 antibody could inhibit the binding of the 70-kDa amino terminus to the 160-kDa substrate by ≥ 90% even though these antibodies recognize exclusive epitopes in the type III\textsubscript{12–14} and type III\textsubscript{1} repeats, respectively (23, 40). Because this finding seems to be incompatible with the simple notion that the heparin II domain and the III\textsubscript{1} repeat represent two independent I\textsubscript{1–5} binding sites, we suggest that binding of the I\textsubscript{1–5} repeats to the 160-kDa substrate involves cooperative interactions between the III\textsubscript{12–14} and the III\textsubscript{1} repeats.

Conceivably, such cooperative interactions might depend on the III\textsubscript{1} and III\textsubscript{12–14} repeats coming into close contact with each other. Thus, binding of type I\textsubscript{1–5} repeats would be inhibited by either mAb 9D2 or IST-2, whereas an antibody like mAb C\textsubscript{6}F\textsubscript{10} (23) would have little or no effect because it recognizes an epitope in the III\textsubscript{8–11} repeats. Binding of I\textsubscript{1–5} repeats would also be expected to be completely and specifically inhibited by the heparin II or 44-kDa fragments because they could readily disrupt the apposition of III\textsubscript{12–14} and III\textsubscript{1} repeats in the 160-kDa substrate. Apposition of III\textsubscript{1} and III\textsubscript{12–14} repeats might be facilitated by the highly electronegative decapeptide in fibronectin (residues 722–731) in the type III\textsubscript{2} repeat (41) because this decapeptide, like heparin, could bind to heparin binding sequences in the III\textsubscript{12–14} repeats (42, 43). In intact fibronectin, apposition of the III\textsubscript{12–14} and III\textsubscript{1} repeats may also involve the binding interaction between the III\textsubscript{1} and III\textsubscript{12–14} repeats described recently by Ingham et al. (44).

Because the heparin II domain falls in between integrin binding sites, its activity may well be regulated through tensile forces created by integrin-mediated mecanochemical interactions between the cytoskeleton and fibronectin dimers (13). Such interactions are thought to promote reversible unfolding of one or more type III repeats (16) resulting in the exposure of self-association sites within fibronectin. The “activated” heparin II domain could then bind directly to the I\textsubscript{1–5} repeats and/or facilitate I\textsubscript{1–5} binding through cooperative interactions with another type III repeat, such as III\textsubscript{1}.

Our evidence for direct binding interactions between I\textsubscript{1–5} and III\textsubscript{12–14} repeats lends further support to the notion that the assembly of a complex fibronectin matrix requires multiple I\textsubscript{1–5} binding sites in fibronectin. As predicted before (5, 45), single binding interactions of the I\textsubscript{1–5} repeats can only give rise to uniform fibrils, whereas additional interactions would also promote fibril thickening and branching and thereby add considerable complexity and flexibility to a fibronectin matrix. The availability of multiple I\textsubscript{1–5} binding sites in fibronectin could also reconcile conflicting views of the role of the III\textsubscript{1} repeats in matrix assembly. For example, it now seems possible that the assembly of fibronectin dimers lacking the III\textsubscript{1–7} repeats (26) in fibrils could proceed entirely via I\textsubscript{1–5}-III\textsubscript{12–14} interactions.

Although our studies clearly attest to the importance of III\textsubscript{12–14} as well as the III\textsubscript{1} repeats in I\textsubscript{1–5}-dependent fibronectin-fibronectin binding interactions during fibrillogenesis, it is also evident that binding of the 70-kDa amino terminus to cell layers can occur independently of these two type III domains. As we have shown before (12), the 70-kDa fragments of fibronectin can bind to cycloheximide-treated cells in the absence of any III\textsubscript{1} or III\textsubscript{12–14} repeats. Such binding could not be attributed to any residual fibronectin left on the cell surface because the same cells failed to bind any 70-kDa fragments when they were plated on collagen or vitronectin. In addition, we have shown here that mAbs IST-2 and 9D2, even when used together, fail to inhibit binding of 70-kDa fragments to cycloheximide-treated cells. The inability of these antibodies to block binding of the amino terminus to cell layers cannot be attributed to an accessibility of their epitopes because these antibodies were added at the time cells were plated and thus had ample access to the substrate (12). Interestingly, the antibodies were not entirely ineffective in cell layers but actually enhanced binding of 70-kDa fragments by ~40%. Such enhancement is seen not only in our cycloheximide-treated cells, but also in normal fibroblast cultures treated with anti-III\textsubscript{1} Fab' fragments (23). The fact that the antibodies fail to block binding of the amino terminus to cell layers may seem in conflict with our observation that fibronectin fragments can inhibit such binding effectively. In this case, however, the fragments,

![Graph showing binding of 125I-75-kDa fragments to cell layers is not inhibited by the mAbs IST-2 and 9D2. Binding of the 125I-labeled 70-kDa fragments to cell layers () and to substrate alone (●) was measured at increasing concentrations of mAb 9D2 in the presence of a fixed amount of mAb IST-2 (1.6 μg/ml). Suspensions of cycloheximide-treated cells were mixed with mAb 9D2 and/or mAb IST-2 and plated in culture wells that had been coated with 3.2 pmol/well of the 160-kDa cell adhesion fragment. Cell attachment and spreading were unaffected by any of the antibody treatments (not shown). After 3 h, the confluent monolayers (3 × 10\textsuperscript{4} cells/well) were labeled for 1 h with 125I-labeled 70-kDa fragments (2 × 10\textsuperscript{5} cpm/well or 0.1 pmol/well). Binding to substrate alone was measured at the same time. Data represent means of triplicate measurements of specific binding normalized to specific binding seen in the absence of antibodies (0.5 fmol/well in the presence or absence of cells). Bars represent the S.E.](http://www.jbc.org/)

[Figure 11: Binding of 125I-75-kDa fragments to cell layers is not inhibited by the mAbs IST-2 and 9D2. Binding of the 125I-labeled 70-kDa fragments to cell layers (○) and to substrate alone (●) was measured at increasing concentrations of mAb 9D2 in the presence of a fixed amount of mAb IST-2 (1.6 μg/ml). Suspensions of cycloheximide-treated cells were mixed with mAb 9D2 and/or mAb IST-2 and plated in culture wells that had been coated with 3.2 pmol/well of the 160-kDa cell adhesion fragment. Cell attachment and spreading were unaffected by any of the antibody treatments (not shown). After 3 h, the confluent monolayers (3 × 10\textsuperscript{4} cells/well) were labeled for 1 h with 125I-labeled 70-kDa fragments (2 × 10\textsuperscript{5} cpm/well or 0.1 pmol/well). Binding to substrate alone was measured at the same time. Data represent means of triplicate measurements of specific binding normalized to specific binding seen in the absence of antibodies (0.5 fmol/well in the presence or absence of cells). Bars represent the S.E.](http://www.jbc.org/)
 unlike the antibodies, could simply interact with the ligand in solution.

In conclusion, the heparin II domain in fibronectin, like the III1 repeat, plays an important role in fibronectin fibrillogenesis but does not appear to participate in all I1-5-dependent steps in matrix assembly. The III12-14 repeats may participate in fibrillogenesis by binding directly to the amino terminus and/or facilitate binding of the amino terminus through cooperative interactions with the III1 repeat.

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