Divalent Cation Modulation of Fibronectin Binding to Heparin and to DNA*

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Fibronectin is an adhesive glycoprotein that binds to heparin and to DNA. The binding of tryptic fragments of human plasma fibronectin to these ligands is found to be highly dependent on the concentration of divalent cations. We have identified 3 types of binding to heparin. 1) Calcium-sensitive binding is inhibited by CaCl₂, but not by MgCl₂ or by MnCl₂. The NH₂-terminal 31,000-dalton fragment (fragment 23) has this type of binding, which is half-maximally inhibited by 3 to 4 mM CaCl₂. 2) Divalent cation-sensitive binding is exhibited by a 75,000-dalton fragment (fragment 13); its binding is inhibited by all 3 divalent cations. 3) Divalent cation-insensitive binding is characteristic of a 95,000-dalton fragment (fragment 23) has this type of binding, which is half-maximally inhibited by 3 to 4 mM CaCl₂. The binding of tryptic fragments (fragments 10, 13, and 23) are not disulfide-bonded to other fragments. Specific tryptic fragments of fibronectin also bind readily to native DNA in the presence of EDTA, but the binding of all fragments is abolished by the presence of 10 mM CaCl₂ or MgCl₂. Our results indicate that the binding of specific domains of fibronectin to heparin or to DNA can be modulated by divalent cations.

Fibronectins are adhesive, high molecular weight glycoproteins located on the cell surface, in extracellular matrices, or in plasma. They are thought to function in cell-substratum and cell-cell adhesion, maintenance of normal morphology of cells, cell migration, wound healing, phagocytosis and reticuloendothelial clearance, and blood coagulation (1-8).

The initial molecular event in these functions appears to be specific binding of fibronectin to a second biological macromolecule such as collagen, heparin and other glycosaminoglycans, fibrin, or plasma membrane components (1-8). Subsequent covalent cross-linking to some of these molecules can occur by a transglutaminase reaction (9-11). Fibronectin can also bind to 2 intracellular molecules, DNA (12) and actin (13, 14), although the physiological significance of these interactions is not yet known. These multiple ligand-binding activities have been localized to specific protease-resistant domains on the fibronectin molecule; at least 5 to 6 structural and functional domains have been identified (15).

The complex of fibronectin and heparin may play an important role in binding collagen and in phagocytosis during reticuloendothelial clearance of colloids. Heparin promotes the binding of fibronectin to collagen (16-18). In addition, fibronectin and heparin stimulate the phagocytosis of gelatin by liver slices and murine macrophages (7, 19-21) and the binding of type III collagen to macrophages (22), and they also induce a cryoprecipitate with fibrinogen in plasma (23).

Fibronectin binding to heparin occurs with moderately high affinity (KD = 10⁻⁹ to 10⁻⁷ M), but Scatchard analysis shows that the binding is not simple and suggests the existence of at least 2 components (24). Fibronectin binds to DNA with significantly lower affinity (KD = 5 × 10⁻⁶ M) (12). We have recently purified a heparin-binding domain of fibronectin (24, 25). However, 1 or more additional heparin-binding sites have been identified by Hakomori and co-workers (26, 27), Richter et al. (28), and us (29). The characteristics and functional relationships of each of these binding sites remain unclear. In this study, we describe differences between the binding of specific fibronectin fragments to heparin and to DNA, and describe 3 classes of sensitivity to divalent cations. The identification of these differences should facilitate the purification of each separate heparin-binding site, and their existence suggests a possible means of ionic modulation of fibronectin interactions with other macromolecules.

EXPERIMENTAL PROCEDURES

Materials and Methods

Preparation of Fibronectin—Fibronectin was purified from human plasma by gelatin and heparin affinity column chromatography at room temperature (23 °C) unless otherwise specified. Human plasma from the National Institutes of Health Blood Bank (500 ml) was supplemented with 12.5 ml of 0.2 M EDTA and 2.5 ml of 0.2 M phenylmethanesulfonyl fluoride (Sigma) freshly prepared in 95% ethanol. After centrifugation at 9000 × g for 15 min at 4 °C, the supernatant material was incubated at 37 °C for about 10 min, desalted for 5 min, and applied to a precolumn of Sepharose CL-4B (bed volume of 20 ml) which had been equilibrated with 0.15 M NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4). The flow-through fractions were applied to a gelatin-Sepharose affinity column (bed volume of 20 ml) which had been washed with 40 ml of 4 mM Tris-HCl (pH 7.4), followed by 60 ml of 0.15 NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4). After washing with 40 ml of 0.5 M NaCl, 10 mM Tris-HCl (pH 7.4), followed by 60 ml of 0.15 NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4), the gelatin-Sepharose column was eluted by 40 ml of 0.15 NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4), the gelatin-Sepharose column was eluted by 40 ml of 0.15 NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4), the gelatin-Sepharose column was eluted by 40 ml of 0.15 NaCl, 5 mM EDTA, 10 mM Tris-HCl (pH 7.4), and was stored in polypropylene tubes (Nunc) in liquid N₂ at 4 to 19 mg/ml.

The gelatin and heparin affinity columns can be used repeatedly, and were stored at 4 °C after the addition of 0.02% sodium azide. The...
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heparin affinity chromatography step was necessary to remove trace contaminants that remain after gelatin affinity chromatography (30, 31). About 85 mg of pure fibronectin were obtained from 500 ml of human plasma.

Trypsin Digestion—Fibronectin (3 mg/ml) was digested by L-3,4-dihydroxyphenylalanine substituted trypsin ( Worthington, 238 units/mg) in 1 mm CaCl2, 30 mm NaCl, 50 mm Tris-HCl (pH 7.0) in polypropylene tubes (Nunc or Falcon) at 30 °C at the specified enzyme-substrate ratios. At specified intervals, digestion was terminated by the addition of 1/200 volume of 0.2 M phenylmethylsulfonyl fluoride freshly prepared in 85% ethanol and immersion in an ice bath. The same amount of phenylmethylsulfonyl fluoride was added 1 h later. The digest was frozen in powdered dry ice and stored at −80 °C.

Binding Analysis by Affinity Chromatography—Tryptic digests of fibronectin (80 μg) in 0.2 ml of Tris- NaCl (50 mm Tris-HCl (pH 7.0), 0.1 m NaCl) containing 10 mm EDTA or divalent cations were applied to heparin-Sepharose affinity columns (0.7 x 4 cm) (Bio-Rad polypropylene Econo-Columns or Isolab Quik-Sep columns) of 0.25 ml bed volume that were prewashed with 0.5 ml of 0.5 m NaCl, 50 mm Tris-HCl (pH 7.0) followed by 2 x 0.5 ml of Tris-NaCl containing 10 mm EDTA or divalent cations. The columns were then washed with 2 x 0.5 ml of the above solutions and eluted as described in the figure legends by 2 x 0.5 ml of Tris-NaCl containing 10 mm MgCl2 or 10 mm CaCl2, or alternatively by 0.5 m NaCl. 50 mm Tris-HCl (pH 7.0) to elute all bound fragments. Analysis of 10 samples usually required about 30 min from application of samples to elution of binding fragments. The eluates were collected in glass test tubes (borosilicate glass, 10 x 75 mm, Kimble) and were adjusted to a final concentration of 9% (w/v) sucrose, 0.1% sodium dodecyl sulfate, 10 mm sodium phosphate (pH 7.0), 0.0015% bromphenol blue.

Diagonal SDS-Polyacrylamide Gel Electrophoresis—Disulfide linkages between fragments were examined by 2-dimensional gel electrophoresis. To obtain a full range of fragments, 80 μg of each of 3 tryptic digests of fibronectin were combined after digests at enzyme-substrate ratios and times of 0.002% for 30 min, 0.2% for 30 min, and 2% for 30 min. The mixture was electrophoresed in a 1.2-mm thick SDS-polyacrylamide slab gel without reduction by dithiothreitol. After electrophoresis in the first dimension, a 1-cm gel strip was cut off with a circular blade and incubated in 50 ml of 0.1 m dithiothreitol, 0.5% SDS, 0.01 m sodium phosphate (pH 7.0) with gentle swirling at 23 °C for 40 min. The strip was placed horizontally on the top of a second dimensional SDS-polyacrylamide slab gel that was prepared as described previously (28). Heparin-Sepharose was prepared according to Fujikawa et al. (35) and native DNA-cellulose was purchased from Enzo Biochem. Protein was determined by the method of Lowry et al. (36) with bovine serum albumin standards.

RESULTS

Digestion by trypsin of highly purified human plasma fibronectin generates the series of fragments shown in Fig. 1A. After testing 18 variations of enzyme concentration and time, 8 conditions were chosen to provide a representative series of tryptic digests (Fig. 1A), and the 29 major tryptic fragments of fibronectin were numbered according to their relative mobilities in SDS-polyacrylamide gel electrophoresis after reduction of disulfide bonds.

The binding of these fibronectin fragments to heparin-Sepharose is markedly dependent on the concentration and type of divalent cation (Fig. 2). The overall binding of fragments decreases in the sequence 10 mM EDTA > 10 mM MgCl2 = 10 mM MnCl2 > 10 mM CaCl2. In the presence of 10 mM EDTA, 19 of 27 fragments bind to heparin; fragments 12, 16-18, 22, 24, 28, and 29 do not bind (Fig. 2).

The presence of divalent cations results in specific inhibition of the binding of certain fragments to heparin. In the presence of either 10 mM MgCl2 or MnCl2, 4 additional fragments are not bound by heparin (Fig. 2; fragments 13, 14, 19, and 26). In addition, 4 other fragments (fragments 20, 23, 25, and 27) show decreased binding. In the presence of 10 mM CaCl2, 2 additional fragments pass through the columns without binding (fragments 23 and 25), and 2 others are less tightly bound (fragments 15 and 20). Twelve fragments (fragments 2-4, 6-11, 16, 20, and 27) are insensitive to divalent cations and continue to bind to heparin in their presence (Fig. 2).

We examined whether these effects of divalent cations might result from simple charge effects by comparing the binding of these fragments to DNA, another negatively charged polymer that is known to bind fibronectin and some fibronectin fragments (12, 29). Unexpectedly, none of the 27 tryptic fragments binds to native DNA-cellulose in the presence of either calcium or magnesium (Fig. 2). A number of fragments do bind in the presence of EDTA, a compound that was routinely included in previously published reports of fibronectin binding to DNA (Fig. 2). The fragments that bind to DNA in the presence of EDTA are similar to but not identical with the fragments binding to heparin in EDTA (Fig. 2).

The presence of 10 mM MnCl2 inhibits the binding of most fragments, but fragments 7 to 10 still bind to the DNA. These patterns of tryptic fragments binding to DNA are quite different from those to heparin, e.g. in the different effects of MnCl2 and MgCl2. The modulation of binding by divalent cations is therefore probably not the result of simple charge effects.

The preceding binding experiments do not indicate whether divalent cations modulate the overall binding of specific fragments to ligands or modulate only their initial recognition and binding interactions. To examine this question, tryptic fragments of fibronectin were bound to heparin-Sepharose in the presence of EDTA and were sequentially eluted by adding 10 mM MgCl2, 10 mM CaCl2, and then 0.5 mM NaCl to the elution buffer.

In the presence of 10 mM EDTA, at least 21 of 29 fragments are bound (fragments 12, 16-18, 21, 22, 24, and 29 are not bound to heparin, Fig. 1B). Treatment with 10 mM MgCl2 (Fig. 1C) results in nearly quantitative elution of 4 fragments (fragments 10, 13, 14, and 27) and partially decreased binding of 9 others (fragments 6-8, 19, 20, 23, 25, 26, and 28).

Subsequent treatment with 10 mM CaCl2 results in a striking release of most fragment 23 with a slight release of 9 others (fragments 2-6, 13, 14, 25, and 27, Fig. 1D). The remainder of the fragments (fragments 1-11, 15, 19, 20, 23, 25, and 27) is eluted in 0.5 mM NaCl (Fig. 1E). These patterns of elution by magnesium and calcium are generally the converse of the patterns of binding in Fig. 2, indicating that these cations inhibit all of the binding interactions of a fragment with heparin, rather than simply preventing its initial recognition and binding interaction with heparin. The release of small amounts of some fragments with each elution, as noted above, suggests weak binding or slightly overlapping specificities, since the elutions were performed at each step using 4 column volumes of all solutions. If the order of elution was reversed, i.e. calcium before magnesium, the pattern of fragments eluted in calcium was the summation of the fragments eluted in Fig. 1, C and D (data not shown). Summarizing the major results
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Fig. 1. Tryptic fragments of fibronectin and their specific elution from heparin affinity columns. A, SDS-polyacrylamide gel electrophoresis of human plasma fibronectin and its tryptic fragments after reduction with 0.1 M dithiothreitol. Fibronectin (3 mg/ml) in 1 mM CaCl₂, 30 mM NaCl, 50 mM Tris-HCl (pH 7.0) was digested at 30 °C by trypsin at enzyme/substrate ratios (w/w) of 0.002% for 30 min (a), 0.02% for 5 min (b), 0.02% for 1 h (c), 0.2% for 30 min (d), 2% for 15 min (e), 2% for 30 min (f), 2% for 6 h (g), and 2% for 24 h (h). 0 indicates fibronectin before initiation of digestion. 80 μg of fibronectin or fibronectin fragments were loaded into each slot. f1, f2, etc. indicate fragment numbers. B-E, 50 μg of the same digests shown in A were applied to two heparin-Sepharose columns in 10 mM EDTA, 0.1 M NaCl, 50 mM Tris-HCl (pH 7.0). Bound fragments were then eluted from one of the affinity columns by 0.5 M NaCl in 50 mM Tris-HCl (pH 7.0) are shown in B. The bound fragments selectively eluted from the second column by 10 mM MgCl₂, 0.1 M NaCl, 50 mM Tris-HCl (pH 7.0) are shown in C; fragments subsequently eluted from the same column by 10 mM CaCl₂, 0.1 M NaCl, 50 mM Tris-HCl (pH 7.0) are shown in D; and the remaining fragments eluted by 0.5 M NaCl, 50 mM Tris-HCl (pH 7.0) are shown in E. It should be noted that the summation of the sequential eluates C, D, and E is equivalent to elute B containing total bound fragments. The letters a, b, c, etc., at the top indicate the specific digests described in A.

Fig. 2. Binding of tryptic fragments of fibronectin to heparin and to native DNA in the continual presence of divalent cations. 27 μl of trypsin digests (80 μg) of fibronectin (0.2% enzyme/substrate ratio for 1 h, a condition chosen to maximize the recovery of fragments shown in Fig. 1) were added to 173 μl of 0.1 M NaCl, 50 mM Tris-HCl (pH 7.0) containing 10 mM EDTA (E), 10 mM CaCl₂ (Ca), 10 mM MgCl₂ (Mg), or 10 mM MnCl₂ (Mn). These aliquots were applied to heparin-Sepharose or DNA-cellulose columns pre-equilibrated with 0.1 M NaCl, 50 mM Tris-HCl (pH 7.0) containing 10 mM EDTA or each divalent cation to examine for the binding of fragments to heparin and to native DNA as described under "Experimental Procedures." After loading, columns were washed with the same solutions containing 10 mM EDTA or the appropriate divalent cation. Bound fragments were then eluted for analysis using 0.5 M NaCl, 50 mM Tris-HCl (pH 7.0). D indicates the original digest (80 μg) loaded on the column; f indicates the number of the fragment.

Fig. 3. Diagonal SDS-polyacrylamide gel electrophoresis of tryptic fragments of fibronectin. 80-μg aliquots from each of digests a, d, and f shown in Fig. 1A were combined and subjected to electrophoresis without reduction (first dimension). A strip of the first dimensional gel was reduced with 0.1 M dithiothreitol for 40 min and then subjected to electrophoresis in the second dimension. Top and left gels were parallel single-dimensional gels of the same sample (a mixture of 13 μg each of digests a, d, and f) without and with 0.1 M dithiothreitol, respectively. The numbers on both sides indicate the fragment numbers.

In these experiments, there are at least 3 classes of fibronectin fragments that bind to heparin: calcium-sensitive (e.g. fragment 23); divalent cation-sensitive (e.g. fragments 13 and 14); and divalent cation-insensitive (e.g. fragments 1-11 and 15).

Since the binding of fibronectin to a ligand can be inhibited by the reduction of disulfide bonds (37), the disulfide bonds of fragments were not reduced before ligand-binding analyses. Some fragments that appear to bind to heparin may therefore be adsorbed to affinity columns because they are disulfide-bonded to other fragments that contain heparin-binding sites. The extent of disulfide bonding between fragments was ex-
amined by 2-dimensional gel electrophoresis before and after reduction of disulfide bonds.

Fig. 3 shows that most of each of the fragments of fibronectin is located along the diagonal, indicating the absence of detectable interfragment disulfide bonds. However, at least 6 fragments (fragments 19, 20, 24, 27–29) are extensively disulfide bonded, and several others show evidence of disulfides in a subpopulation of fragments of that size (Fig. 3). It is therefore difficult to unambiguously interpret the binding of such fragments to affinity columns as evidence for a binding site. However, 3 major fragments analyzed in this study (fragments 10, 13, and 23) show no evidence for such complicating inter-subunit disulfide bonds.

Since fragment 23 is unique in its sensitivity to calcium, we examined its binding at physiological concentrations of this cation. As in unfractionated digests, purified fragment 23 binds to heparin in 10 mM EDTA-Tris-NaCl, but does not bind in 10 mM CaCl2-Tris-NaCl (Fig. 4). A dose-response curve shows that the concentration of calcium required to inhibit the binding of fragment 23 by 50% is 3 to 4 mM (Fig. 4), which is similar to the calcium concentration of 2 to 3 mM in human blood. Parenthetically, it should be noted that even in 10 mM EDTA, but with 0.1 M NaCl present, the binding of fragment 23 to heparin in these experiments is not strong. After very extensive washing (12 or more column volumes), most of fragment 23 is eventually washed off the heparin-Sepharose columns.

**DISCUSSION**

Our four major findings are: 1) different tryptic fragments of fibronectin have markedly different divalent cation sensitivities in binding to heparin; 2) the major types of binding interactions are calcium-sensitive, divalent cation-sensitive, or divalent cation-insensitive binding; 3) the binding to heparin of a calcium-sensitive fragment is inhibited at physiological concentrations of calcium; and 4) the binding of fibronectin fragments to DNA is prevented by divalent cations, and the pattern of sensitivity of specific fragments is different from the pattern for heparin binding.

Our results indicate that the binding of fibronectin to heparin is complex, in agreement with the binding characteristics found previously in studies of the intact molecule (23, 24, 38).

The existence of at least 3 classes of binding interaction suggests that fibronectin has at least 3 different heparin-binding sites. Two-dimensional SDS gel analysis in the absence and presence of reducing agent indicates that at least fragments 10, 13, and 23 can bind to heparin as monomers; these fragments show different cation sensitivities (divalent cation-insensitive, divalent cation-sensitive, and calcium-sensitive, respectively). Peptide mapping using *S. aureus* protease V8 digestion of proteins separated in an SDS-polyacrylamide gel shows that fragment 23 does not share any common peptides with fragments 10 and 13, but that there is considerable but not complete homology between peptides 10 and 13. The calcium-sensitive, heparin-binding site in fragment 23 is therefore distinct from other sites; however, fragments 10 and 13 are probably partially overlapping fragments. Although other interpretations are possible, the simplest interpretation of our data is that there are two additional heparin-binding sites in fragment 10 (Mr=95,000), one of which is insensitive to divalent cations, accounting for the insensitivity of the whole fragment. Further cleavage by trypsin, only the divalent cation-sensitive site is retained in fragment 13 (Mr=75,000) and a divalent cation-insensitive site may be retained in fragment 27 (Mr=21,000), which is insensitive to inhibition by divalent cations. Further structural and sequencing studies are needed to elucidate these relationships.

This study clarifies apparently conflicting results in the previous literature concerning the number of heparin-binding sites on fibronectin (25, 26, 29, 39), since the number of heparin-binding fragments is shown to vary depending upon the divalent cation composition of buffers. Fragment 23 (Mr=31,000), shown in this study to be calcium-sensitive, corresponds to the NH2-terminal domain of fibronectin, i.e. the 32,000-dalton heparin-binding thermolysin fragment from hamster plasma fibronectin reported by Hakomori and co-workers (26, 27) and the 30,000-dalton heparin-binding cathepsin D/plasmin fragment from human plasma fibronectin reported by Richter et al. (28).

The divalent cation-insensitive heparin-binding site can be isolated in either the presence or the absence of calcium (24, 25, 29) and has been localized to the carboxyl-terminal third of the polypeptide chain of fibronectin (24, 38). A 50,000-dalton pronase fragment containing this domain has been purified and characterized (25). The divalent cation-sensitive heparin-binding site may be located in a structural domain at the COOH-terminal end of the 50,000-dalton fragment (15, 29), and the present study suggests that it can now be isolated by appropriate manipulation of the concentrations of divalent cations (Fig. 2, fragments 13 and 14).

Our results suggest that the interaction of fibronectin with heparin (and possibly with the closely related molecule heparan sulfate) may be affected by divalent cations in vivo. Since divalent cation-sensitive and insensitive sites are presumably present on the same polypeptide of intact fibronectin, heparin would be expected to bind by at least one site to fibronectin regardless of the ionic environment; consistent with this notion, the binding of fibronectin to heparin does occur in the presence or absence of divalent cations (24, data not shown). However, the modulation of binding, shown here to occur at physiological concentrations of extracellular calcium, could regulate the interaction of specific sites on fibronectin with specific extracellular glycosaminoglycans, and might alter the strength of binding or the nature of cross-links between extracellular matrix molecules.

Although fibronectin is known to bind to DNA, studies of this binding interaction have generally been performed in the...
presence of EDTA (12). Specific fragments of human and chicken fibronectins bound to native DNA in EDTA (29, 40).

This study confirms these results, but also shows that such binding is abolished in the presence of calcium or magnesium (Fig. 2). About 90% of intact, purified fibronectin bound to a native DNA-cellulose column in the presence of EDTA, whereas 33 to 70% of the fibronectin bound in the presence of these ions. The binding of intact fibronectin to DNA in the presence of divalent cations therefore appears weak, and proteolytic fragmentation appears to weaken the interaction further so that binding can occur only in the absence of divalent cations. It is therefore not clear whether fibronectin binds to DNA with sufficient strength under physiological conditions to be important biologically.

It is of interest to consider the effects of divalent cations on the binding of fragments of plasma fibronectin to other ligands. In contrast to the results with heparin, we could find no specificity in the effects of calcium, magnesium, or manganese ions on the binding of any particular fragment in a mixture of fibronectin fragments to gelatin, actin, fibrin, or S. aureus. However, the total quantity of fragments binding to certain ligands was often slightly increased or decreased, with the amounts retained in the presence of various ions found to be in the following order: MgCl₂ > MnCl₂ = CaCl₂ > EDTA for gelatin-Sepharose (only minor differences among all 4), CaCl₂ = MgCl₂ = MnCl₂ ≥ EDTA for fibrin-Sepharose, and EDTA ≥ MnCl₂ ≥ MgCl₂ > CaCl₂ for both actin-Sepharose and S. aureus cells (data not shown). These results further suggest that the effects of divalent cations on fibronectin interactions with heparin reflect modulation of specific sites on fibronectin, since only certain fragments are affected in their binding to this particular ligand.

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