Characterization of Fibronectin Interactions with Glycosaminoglycans and Identification of Active Proteolytic Fragments*

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Fibronectin is a major cell-surface glycoprotein which has been reported to interact with glycosaminoglycans. A nitrocellulose filter-binding assay was developed to quantitate these interactions at physiological pH and ionic strength. Fibronectin isolated from chick embryo fibroblasts binds both hyaluronic acid and heparin; heparan sulfate is bound less efficiently, and chondroitin sulfate and glycopeptides are bound minimally. The binding of hyaluronic acid and heparin to fibronectin is saturable and reversible and occurs at separate binding sites. The binding of both molecules to fibronectin is not blocked by EDTA or by other glycosaminoglycans, and is only moderately inhibited by elevated ionic strength. Scatchard analyses reveal nonlinear, high affinity binding to fibronectin with a $K_d$ of approximately $10^{-7}$ to $10^{-8}$ M for these glycosaminoglycans. The affinity for heparin was utilized for the isolation of heparin-binding domains of fibronectin on heparin-agarose affinity columns. Heparin-binding proteolytic fragments with apparent molecular weights of 180,000 and 50,000 were isolated following hydrolysis of fibronectin by chymotrypsin or pronase, respectively. The possible involvement of such high affinity binding sites of fibronectin in the binding of glycosaminoglycans to the cell surface or in the organization of extracellular matrices is discussed.

Cellular fibronectin is a major cell-surface glycoprotein synthesized by a variety of cell types. A related glycoprotein termed plasma fibronectin (cold insoluble globulin) is present in blood at 0.3 ng/ml and is structurally and biologically similar, but not identical, to cellular fibronectin. Both forms of fibronectin appear to function as adhesive molecules, affecting a wide variety of cellular events (reviewed in Refs. 1-6).

A series of previous studies established that both forms of fibronectin can interact in some fashion with glycosaminoglycans. Plasma fibronectin was shown to interact with heparin by precipitation and affinity chromatography studies. Heparin binding was stimulated by calcium and apparently abolished by ionic strengths above 0.3 (7). These results suggest that the binding might occur via electrostatic mechanisms. Glycosaminoglycans can also increase or decrease the extent of binding of plasma fibronectin to collagen (8), and polyamines also inhibit its binding to collagen (9).

With respect to cellular interactions with glycosaminoglycans plus fibronectin, it has been suggested that cells of the reticuloendothelial system utilize plasma fibronectin for uptake of denatured collagen (10), and that heparin can modulate this process (11). Cross-linking studies with fibroblastic cells suggest that cellular fibronectin is in proximity to sulfated proteoglycans on the cell surface, suggesting that the cellular form of fibronectin also interacts with proteoglycans (12). All of these previous studies support the concept that glycosaminoglycans can interact with plasma or cellular fibronectin. We have investigated the biochemical characteristics and specificity of such interactions with cellular fibronectin. A filter-binding assay was developed to obtain quantitative information; our studies were usually conducted under physiological salt conditions in attempts to characterize specific interactions that are independent of simple electrostatic attraction. We also describe the isolation of active proteolytic fragments of fibronectin that bind to heparin, and provide models for the organization of these sites relative to other recently isolated binding sites of fibronectin.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cellular fibronectin was purified from chick embryo fibroblasts exactly as described previously (13). The fibronectin was at least 98% pure according to electrophoresis in 4 to 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide slab gels (13, 14). 1H-labeled fibronectin was prepared from chick fibroblasts cultured for 24 h in regular culture medium plus 1 μCi/ml of [1H] glycine (64 μCi/mmol; Amersham).

Highly purified, reference standard grade human umbilical cord hyaluronic acid, bovine lung heparin, sturgeon chondroitin 4-sulfate and chondroitin 6-sulfate, bovine cornea keratan sulfate, and hog mucosal dermatan sulfate were kind gifts from Drs. M. B. Mathews and J. A. Cifonelli, Department of Pediatrics, University of Chicago. Heparin (porcine intestinal mucosa, grade I, 189.7 USP units/mg) was also purchased from Sigma, and shark cartilage chondroitin 6-sul fate was from Miles Biochemicals.

Pro tease-free bacterial collagenase was from Advance Biofactures Corp. (Form III, 181 units/mg); a-chymotrypsin (49.2 units/mg) and bovine testicular hyaluronidase (11,560 units/mg) were from Worthington; pronase (45 units/mg) and Streptomyces hyaluronidase (5,990 units/mg) were from Calbiochem-Behring; and chondroitinase ABC was from Miles Biochemicals. Bovine r-globulins and fibrinogen (96% clottable) were obtained from Miles Biochemicals and fetuin, bovine serum albumin, pepstatin A, N-acetylgalactosamine, phenylmethylsulfonfyl fluoride (PMSF), and glucuronic acid were from Sigma. Sepharose CL-4B and Sephadex G-50 and G-100 were from Pharmacia.

**Standard Binding Assay**—Purified cellular fibronectin was incubated at a final concentration of 275 μg/ml (1.25 x 10^{-10} M monomer) with various 3H-labeled, purified glycosaminoglycans in complete Dulbecco's phosphate-buffered saline (PBS) containing an additional 10 mM sodium phosphate, pH 7.4, in polypropylene test tubes at 23°C. The final volume was usually 0.64 ml, although for certain experiments with material of high specific activity, the assay was scaled down. After 60 min, the samples were agitated on a Vortex mixer, then vacuum-filtered through 2.5-cm diameter 0.45-μm nitrocellulose filters (Millipore, type HA). Residual sample was transferred from

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the tubes with two 1-ml washes of PBS, then the filters were rapidly washed with 4 ml of PBS five times at 23°C. The washing procedure required less than 30 s. Preliminary experiments indicated that the recovery of radioactive heparin or hyaluronate bound to fibronectin was not altered if the washing was performed at 4°C, or if serum-free culture medium (Ham's F-10 medium) was substituted for the PBS. Although heparin was routinely added last to reaction mixtures, the addition of labeled glycosaminoglycans after the fibronectin did not affect the amount of binding even if delayed up to 10 min (the longest period tested).

The filters were placed into 12 ml of Aqasol (New England Nuclear), and digested until clear, and the radioactivity was determined with a Beckman LS-230 scintillation counter with 32% efficiency for tritium, as determined by adding internal standards of 3H-labeled tolue (New England Nuclear).

As will be described below, glycosaminoglycan binding was inhibited by 4 mM NaCl or hyaluronidases in certain cases. This inhibition of binding was not due to artifactual loss of fibronectin from the filters, since recovery of 3H-glucosamine-labeled fibronectin from filters under these conditions was the same as that of untreated controls (100 ± 2%).

Preparation of Radioactive Glycosaminoglycans—Secondary chick embryo fibroblasts were plated at 270 cells/100 mm tissue culture dish (Falcon) and cultured as described previously in 5% heat-inactivated calf serum plus 10% tryptose phosphate in Ham's F-10 medium (13). After 24 h, the medium was changed and 25 µCi/ml of 3H-glucosamine (44 Ci/mmol; New England Nuclear) was added to the standard culture medium containing 1 mM of sodium phosphate, pH 7.4; Buffer A, 0.04 M Tris/Cl, pH 8.0; 0.02 M NaCl or hyaluronidases in certain cases. This inhibition of binding was not due to artifactual loss of fibronectin from the filters, since recovery of 3H-glucosamine-labeled fibronectin from filters under these conditions was the same as that of untreated controls (100 ± 2%).

The medium was incubated for 72 h, and the radioactivity remaining with the glycosaminoglycans was determined by liquid scintillation spectrometry. The glycosaminoglycans were digested with chondroitinase ABC and deproteinized with trichloroacetic acid.

Chondroitnase ABC Digestion of Fibronectin—Fibronectin was labeled with 2 µCi/ml of 3H-glucosamine (55.6 Ci/mmol; New England Nuclear) for 24 h and purified as described (13). The fibronectin was dialyzed against 0.01 M EDTA, 0.01 M Tris/Cl, pH 8.0. Nine-milliliter fractions plus washes were applied to a Sephadex G-50 (superfine) column (0.9 x 60 cm) in PBS, calibrated with the following molecular weight standards detected by the phenolsulfuric acid procedure (19): hyaluronic acid (200,000), dermatan sulfate (400,000), chondroitin 6-sulfate (290,000), and heparin (14,000). The average size of the hyaluronic acid was defined as the apparent molecular weight above or below which half of the total radioactivity in the preparation was present.

[3H]Heparin (0.332 µCi/mg; New England Nuclear) was applied to a Sephadex G-10 column (0.9 x 60 cm) equilibrated with PBS. The high molecular weight peak fractions were pooled as described below; an aliquot was rechromatographed on the same column to calibrate size, and the remainder was stored at −20°C and used for binding studies. The column was calibrated with heparin (Mw = 14,000), dextran T10 (5,200), dextran T40 (28,000), and sucrose (34,200). [3H]-labeled proteoglycans (A1-D1 fraction) were prepared by Dr. John Pennypacker, National Institute of Dental Research, from mouse articular cartilage incubated with [3H]glucosamine exactly as described (20, 21).

Binding Affinities—Increasing amounts of acid-labile (439 cpm/pmol of hyaluronic acid) or [3H]heparin (1,250 cpm/pmol of heparin) were added to the standard assay at final concentrations of 0.1 ng/ml up to 100 ng/ml in 0.15 M NaCl. This analysis was performed (22). The results were confirmed by using mixtures of reference standard grade unlabeled heparin or hyaluronic acid and labeled material, and the amounts of binding were calculated by accounting for the dilution of the radioactivity by unlabeled material.

Chondroitnase ABC-Induced Degradation of Fibronectin—Heparin was detected by the carbohydrate reaction for uronic acid (18).

Isolation of Heparin-binding Fragments—Heparin (Sigma) was coupled to agarose via cyanogen bromide as described (25). A trace amount of proteolytic activity remaining with the heparin was inhibited by preincubating the heparin-agarose with 2 µM FMRFamide, 5 µM N-acetyltyrosine, 5 µM pepstatin in Buffer E (0.1 M NaCl, 10 mM CaCl2, 50 mM Tris/Cl, pH 7.0) for 24 h at 23°C, followed by exhaustive washing with Buffer E. The isolated heparin was then used as a carrier for the isolation of chondroitinase ABC digestion products. The columns were then washed with at least 10 volumes of Buffer E. Preliminary digestions of the bound fibronectin were performed directly on the columns by applying 0.5 ml of various proteases in Buffer E. a-Chymotrypsin was at either 0.5 or 5 µg/ml, and pronase was at 5 µg/ml. After 1 h at 23°C, the columns were washed with 10 volumes of Buffer E containing 2 mM FMRFamide. The gel beads were transferred to glass tubes containing 10 ml of Buffer E containing 50 mM Tris/Cl, pH 7.0. The filters were washed with 0.1 ml of 6 M SDS, 10 mM NaCl, 50 mM Tris/Cl, pH 7.0, and centrifuged to remove insoluble material, and the supernatant was applied to a Sephadex G-50 column as described for the chondroitinase ABC digestion. The completeness of the reaction was confirmed by demonstrating the degradation of carrier heparin, which was detected by the carbazole reaction for uronic acid (18).
gel beads were then sedimented and the supernatants were examined on polyacrylamide gels after reduction with 0.1 mM dithiothreitol (13, 14).

In certain experiments, fibronectin that was bound to heparinagarose was subjected to sequential digestions with chymotrypsin and pronase. After 1 h in 5 μg/ml of chymotrypsin, the columns were washed with 10 volumes of Buffer E, then incubated an additional 1 h in 5 μg/ml of pronase, washed, and eluted with boiling SDS as described above. In two experiments, the column eluates after 5 μg/ml of chymotrypsin digestion were reapplied to 0.5-ml gelatin-agarose columns, and washed with Buffer E containing 2 mM PMSF after 10 min then, the bound fragments were eluted with boiling 2% SDS as described above. The recovery of a pronase-generated 50,000-dalton fragment from heparin-agarose columns was determined by two methods. The amounts of protein originally in fibronectin or in the 50,000-dalton fragments were compared in polyacrylamide gels stained with Coomassie brilliant blue (13, 14) by densitometry with a Joyce-Loebl densitometer. The absorbance was linearly proportional to the amount of protein applied in the ranges utilized.

A second method was to determine the recovery of radioactivity from fibronectin originally labeled for 24 h with [3H]glycine (an abundant amino acid in fibronectin (13)). The [3H]fibronectin was permitted to bind to 1-ml heparin-agarose columns, as described above, and washed with 5 ml of Buffer E, and the beads were transferred to scintillation vials for determination of radioactivity. Parallel columns were subjected to pronase digestion, as described, and washed with 10 ml of Buffer E, and radioactivity was determined. The control column consisted of agarose activated with cyanogen bromide but subjected to coupling conditions in the absence of heparin.

RESULTS

Glycosaminoglycan Binding Assay—Fibronectin is present on the surface of cells at physiological pH as insoluble, relatively immobile aggregates or fibrils (reviewed in Refs. 1, 2, and 5). Although isolated purified cellular fibronectin can be maintained in solution at alkaline pH, it regains its insolvibility at neutral pH (13). This property was used to establish a binding assay in which purified fibronectin is neutralized and incubated with various ligands for 1 h in Dulbecco’s PBS at pH 7.4 prior to filtration through nitrocellulose filters as described under “Experimental Procedures.” Studies with [14C]glycine-labeled fibronectin indicated that all (101 ± 1%) of the input radioactivity was recovered on the filters under the standard incubation conditions. Since 3H-labeled glycosaminoglycans were not retained by these filters, their binding to fibronectin can be detected by measuring the radioactivity that remains bound to filters in the presence of unlabeled fibronectin.

We first examined the binding of [3H]hyaluronic acid to fibronectin. The binding was maximal at 200 to 275 μg/ml of fibronectin (Fig. 1). All subsequent studies were consequently performed with 275 μg/ml of fibronectin (1.25 × 10^-6 M monomer, if reduced; the molecule consists primarily of disulfide-bonded dimers and multimers).

The time course of [3H]hyaluronic acid binding is shown in Fig. 2. Binding reaches equilibrium in approximately 10 to 15 min; the routine assay involves an incubation of 60 min. The binding is reversible after dilution of reaction mixtures with PBS, and the process requires approximately 2 h (Fig. 3). The presence of unlabeled ligand increases the rate of release of labeled ligand from fibronectin (Fig. 3). Similar binding and reversibility results were obtained with [3H]heparin, except that heparin binding reached equilibrium within 30 to 60 s (data not shown).

This binding of hyaluronic acid isolated from culture medium to fibronectin was compared to the binding of [3H]-hyaluronic acid isolated from the corresponding cell layers following pronase digestion. Cell layer hyaluronate was also bound by fibronectin, although the percentage of total radioactivity bound was lower for cell-associated than for medium hyaluronate from the same cell cultures (36 ± 4% versus 58 ± 3%, respectively; specific activities were 10,030 versus 2,130 cpm/nmol of uronic acid). We therefore utilized hyaluronic acid purified from culture medium for all subsequent experiments.

The specificity of glycosaminoglycan binding to fibronectin was examined with purified glycosaminoglycans isolated from chick embryo fibroblasts. Cultures were labeled for 24 h with [3H]glucosamine, then subjected to pronase digestion and DEAE-cellulose column chromatography as described under “Experimental Procedures.” Hyaluronic acid isolated from culture medium was the glycosaminoglycan most effectively

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**Fig. 1 (left).** Binding of hyaluronic acid by fibronectin. The binding of hyaluronic acid by fibronectin in PBS for 60 min at 23°C, then, the mixtures were collected on nitrocellulose filters and the retained radioactivity was determined. The specific radioactivity of hyaluronic acid was 431 cpm/nmol of uronic acid, and the maximal binding of input radioactivity was 45%. Values represent means ± S.E. of triplicate samples.

**Fig. 2 (center).** Time course of hyaluronic acid binding to fibronectin. The hyaluronic acid (2,000 cpm; 2,130 cpm/nmol of uronic acid) was incubated with 1.25 × 10^-6 M fibronectin in 0.64 ml of PBS at 23°C and collected on nitrocellulose filters at the indicated times.

**Fig. 3 (right).** Reversibility of hyaluronic acid binding to fibronectin. After incubation for 60 min with 2,000 cpm (2,130 cpm/nmol of uronic acid) of hyaluronic acid plus 1.25 × 10^-6 M fibronectin, the mixture was diluted 1:15 or 1:100 with PBS in polyethylene tubes and further incubated at 23°C for the times indicated prior to collection on filters.
bound by fibronectin (Fig. 4). The proportion of original radioactivity bound varied from preparation to preparation depending on the specific radioactivity of labeling of the hyaluronic acid (e.g. 23% in Fig. 4 and up to 70% binding in other experiments).

Heparan sulfate was also bound by fibronectin, but the largest amount bound was only 5% of heparan sulfate input radioactivity; this low extent of binding does not appear to be due to a low specific activity, since the specific radioactivity of the heparan sulfate used for Fig. 4 was four times that of the hyaluronic acid. Chondroitin sulfate, either from cell monolayers or from medium, bound poorly to fibronectin, and glycopeptides also did not bind (Fig. 4). In experiments described below, the glycosaminoglycan heparan was also found to bind effectively to fibronectin (up to 85% binding).

Two other procedures were compared to the filter-binding assay for evaluating the binding of chick fibroblast glycosaminoglycans to fibronectin. Although both yielded results similar to those of the filter-binding experiments, the amounts of binding were less and the background binding was greater in these assays. The first assay utilized affinity columns of fibronectin coupled covalently to agarose via cyanogen bromide as described (27). Purified [3H]-labeled glycosaminoglycans that remained bound after extensive washing with PBS were eluted with 8 M urea and quantitated in a scintillation counter. The second assay involved incubating fibronectin with glycosaminoglycans for 1 h as for the filter assay, but the fibronectin was, instead, collected by immunoprecipitation with affinity-purified anti-fibronectin antibodies as described (28). Using these assays and the glycosaminoglycans described in the legend to Fig. 4, we found 5.1 and 1.8% binding of hyaluronic acid by the column and immunoprecipitation techniques, respectively, 1.6 and 1.9% binding of heparan sulfate, 0.3 and 0.2% binding of chondroitin sulfate, and 0 and 0% binding of glycopeptides. Because the filter-binding assay was more convenient and reliable, and bound more ligand than either of these latter assays, it was employed for all subsequent experiments.

**Competitive Inhibition Experiments**—The binding of [3H]hyaluronic acid to fibronectin was completely inhibited by the addition of unlabeled hyaluronic acid to reaction mixtures, but not by the inclusion of unlabeled heparin or chondroitin sulfate (Fig. 5) or by other glycosaminoglycans (Table 1). Half-maximal inhibition of [3H]hyaluronic acid binding occurred at approximately 0.25 μg/ml of unlabeled hyaluronate (Fig. 5); at this point, 0.1 μg of [3H]hyaluronate was

**Table I**

<table>
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<th>Condition</th>
<th>cpm bound ± S.E.</th>
<th>Percentage of control</th>
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<tr>
<td>Control</td>
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<tr>
<td>Heparan sulfate (100 μg/ml)</td>
<td>1447 ± 13</td>
<td>102</td>
</tr>
<tr>
<td>Keratan sulfate (100 μg/ml)</td>
<td>1264 ± 9</td>
<td>88.8</td>
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<tr>
<td>Dermatan sulfate (100 μg/ml)</td>
<td>1482 ± 13</td>
<td>104</td>
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<td>Chondroitin sulfate (100 μg/ml)</td>
<td>1442 ± 49</td>
<td>99.9</td>
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<tr>
<td>NaCl (1 M)</td>
<td>1447 ± 29</td>
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<td>NaCl (4 M)</td>
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<td>EDTA (10 mM)</td>
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<td>N-acetylgalactosamine (0.1 M) + glucuronic acid (0.1 M)</td>
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<tr>
<td>Trypsin (0.1 mg/ml)</td>
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**Fig. 5**. Competitive inhibition of [3H]hyaluronic acid binding to fibronectin by unlabeled glycosaminoglycans. The standard 60-min binding assay was performed with 2,000 cpm of [3H]hyaluronate at 2,130 cpm/nmol of uronic acid plus 1.25 × 10^{-6} M fibronectin in the presence of the indicated final concentrations of unlabeled hyaluronic acid (HA), chondroitin 4-sulfate (CS), keratan sulfate, heparin (H), dermatan sulfate (DS), or heparin (H), dermatan sulfate (DS). unlabeled glycosaminoglycans provided by Drs. Mathews and Cifonelli. The fibronectin was added last to the 0.64-ml reaction mixtures. The closed triangle indicates binding when testicular hyaluronidase (20 μg/ml) was present in the reaction mixtures.

**Fig. 4**. Specificity of glycosaminoglycan binding to fibronectin. The fraction of total radioactivity added to each assay that was recovered on nitrocellulose filters after a 60-min incubation at 23°C with 1.25 × 10^{-6} M fibronectin is indicated for [3H]-labeled glycosaminoglycans or glycopeptides purified as described under “Experimental Procedures”; the sources were chick embryo fibroblast cell monolayers or the media from these cultures labeled with [3H]glucosamine. The preparations had the following specific activities: chondroitin sulfate (cell layer), 2,880 cpm/nmol; heparan sulfate, 3,620 cpm/nmol; chondroitin sulfate (medium), 3,580 cpm/nmol; hyaluronic acid, 425 cpm/nmol of uronic acid. The original quantities of radioactively labeled material per assay and the mean background binding in the controls from which fibronectin was omitted were: chondroitin sulfate (cell layer), 884 and 0 cpm; heparan sulfate, 995 and 5 cpm; glycopeptides (cell layer), 1,716 and 9 cpm; chondroitin sulfate (medium), 1,432 and 8 cpm; glycopeptides (medium), 1,258 and 0 cpm; hyaluronic acid, 552 and 0 cpm. Bars indicate S.E. of triplicate samples.
bound per reaction, and the total concentration of labeled plus unlabeled hyaluronate was 0.74 μg/ml.

In contrast to the results with [3H]hyaluronic acid, the binding of [3H]heparin to fibronectin was inhibited only by unlabeled heparin, with 50% inhibition of binding by 2 μg/ml of unlabeled material, but was not inhibited by unlabeled hyaluronic acid or chondroitin sulfate at even 100 μg/ml (Fig. 6). Its binding may be slightly inhibited by dermatan sulfate at 100 μg/ml, but not by all other glycosaminoglycans tested including heparan sulfate (Table II). The binding sites for hyaluronic acid and heparin are therefore saturable and are functionally separable, since there is no cross-competition for binding at each site by other unlabeled glycosaminoglycans.

**Binding Affinity**—We examined the binding affinities of hyaluronic acid and heparin to fibronectin. These studies were complicated by the broad size ranges of these molecules. For example, the hyaluronic acid chains ranged in molecular weight from 20,000 to at least 500,000 on agarose columns calibrated by glycosaminoglycan standards of known size; the average molecular weight was estimated to be 94,000 (see "Experimental Procedures" for details). Likewise, heparin also has variable chain lengths (Fig. 7); however, since most of the molecules were of a limited size range, we isolated the molecular weight range of 12,000 for binding studies (Fig. 7, Fraction I).

The results of Scatchard analyses are shown in Figs. 8 and 9. The negative reciprocal of the slope is the dissociation constant. For hyaluronic acid, the $K_D \approx 10^{-7}$ M, although the curve deviates slightly from linearity (Fig. 8). For heparin, the curve is biphasic and the estimated $K_D \approx 10^{-7}$ and $4 \times 10^{-9}$. This type of biphasic plot for heparin binding was obtained with three other preparations of fibronectin, as well as in two other experiments in which the concentration of heparin was increased by addition of unlabeled heparin, and the amounts bound were calculated by correcting for the dilution of labeled material. The extrapolated number of binding sites of fibronectin for hyaluronic acid was approximately 0.1/monomer and for heparin was 0.2/monomer of fibronectin. However, it appears likely that these extrapolations may be in error, since the ligand itself probably has multiple fibronectin binding sites due to the repeating disaccharides; consequently, some glycosaminoglycan molecules may bind more than one fibronectin. A direct means of determining the number of binding sites for heparin is described below.

**Inhibitors of the binding of heparin to fibronectin**

Binding of heparin Fraction I to fibronectin was determined as described under "Experimental Procedures" in the presence of the indicated final concentrations of heparin (H; ○—●), hyaluronic acid (HA; △—○), or chondroitin sulfate (CS, ○—○).

**Fig. 6. Competitive inhibition of [3H]heparin binding to fibronectin by unlabeled glycosaminoglycans.** The [3H]heparin-binding assay was performed with 19,000 cpm of [3H]heparin (0.382 mCi/mg; New England Nuclear) in the presence of the indicated final concentrations of heparin (H; ○—●), hyaluronic acid (HA; △—○), or chondroitin sulfate (CS, ○—○).

**Table II**

<table>
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<td>Control</td>
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<td>Heparan sulfate (100 μg/ml)</td>
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<td>Keratan sulfate (100 μg/ml)</td>
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<td>Dermatan sulfate (100 μg/ml)</td>
<td>2549±53</td>
<td>80.2</td>
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<tr>
<td>Chondroitin 6-sulfate (100 μg/ml)</td>
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<td>NaCl (4 M)</td>
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<tr>
<td>Collagenase (0.5 mg/ml)</td>
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**Fig. 7. Gel filtration chromatography of original and purified [3H]heparin.** Commercial [3H]heparin (20 μCi) (●—●) was chromatographed on a Sephadex G-100 column as described under "Experimental Procedures." Fractions I and II were pooled and Fraction I was rechromatographed on the same column to determine size (C—○). Arrows indicate the elution volumes of the molecular weight standards heparin, dextran T40 and T10, and sucrose.

**Fig. 8 (left).** Scatchard analysis of the binding of hyaluronic acid to fibronectin. Equilibrium binding between [3H]hyaluronic acid and 275 μg/ml of cellular fibronectin was determined for 5 to 200 nM hyaluronic acid as described under "Experimental Procedures," and the data were analyzed according to Scatchard (22). We emphasize that this experiment was performed with hyaluronic acid of a mixture of chain lengths. Estimates of affinity and number of binding sites were therefore calculated using the average molecular weight of 94,000; the binding affinity could differ for differing chain lengths.

**Fig. 9 (right).** Scatchard analysis of the binding of heparin to fibronectin. Binding of 50 to 940 nM [3H]heparin (Fraction I) was determined according to Scatchard (22).
sugar (Table I). The binding is also not affected by treatment with purified collagenase, but is sensitive to treatment of reaction mixtures with trypsin or fungal hyaluronidase (Table I).

If the fibronectin is omitted from reaction mixtures, or if it is replaced by equal protein concentrations of fibrinogen, fetuin, bovine serum albumin, or γ-globulin, there is less than 1% nonspecific binding (Table I).

The binding of heparin to fibronectin is more sensitive to salt inhibition than is the binding of hyalurinate (Table II). Approximately 80% of the binding was inhibited by 1 M NaCl and over 90% was inhibited by 4 M NaCl (Table II). However, it is also important to note that binding is highly effective at physiological salt concentrations (standard assay conditions with ionic strength ~0.2), in which 84% of the total amount of [3H]heparin in the reaction mixture is bound to fibronectin.

In addition, we compared the binding of the low molecular weight fraction of commercial [3H]heparin preparations (Fig. 7, Fraction II), and found minimal binding of this fraction to fibronectin (<0.05% of the original radioactivity).

Evaluation for Possible Proteoglycan Contamination—Since proteoglycans are known to bind hyaluronic acid (20), it was important to test for proteoglycan contamination or for covalently bound proteoglycans in our preparations of fibronectin. Fibronectin was labeled with [14C]glucosamine for 24 h as described under "Experimental Procedures," then isolated as usual (11). The fibronectin was then subjected to chondroitinase ABC treatment or to nitrous acid degradation as described under "Experimental Procedures" and examined for the release of radioactivity from possible contaminating proteoglycans. Positive controls for the efficacy of hydrolysis consisted of measuring the degradation of added carrier [3H]glucosamine-labeled proteoglycans or of unlabeled heparin.

Approximately 80% of the binding was inhibited by 1 M NaCl (Table 11). Fraction II), and found minimal binding of this fraction to fibronectin. [3H]glucosamine-labeled fibronectin and [3H]labeled carrier proteoglycans both eluted in the exclusion volume of G-50 column (Fig. 10). After chondroitinase ABC digestion, there was no detectable change in the [3H]fibronectin elution pattern; 95% of the original radioactivity was recovered in the excluded volume fractions. In contrast, the [3H]proteoglycans were degraded by this enzyme, and the radioactivity chromatographed as a retarded peak (Fig. 10). This retarded 3H peak was characterized as dimers by paper chromatography. These results indicate that the fibronectin preparation did not contain detectable amounts of chondroitinase ABC-sensitive proteoglycans of which chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, chondrin, and hyaluronic acid are the glycosaminoglycan constituents.

A mixture of [14C]glucosamine-labeled fibronectin and heparin added as carrier was subjected to nitrous acid degradation. After the reaction, 80% of the starting radioactivity was recovered as a precipitate insoluble in 4 M guanidine. The supernatant was examined for degradation products. By Sephadex G-50 chromatography similar to that shown in Fig. 10, the radioactivity from control or nitrous acid-treated samples appeared only in the excluded fraction. No retarded radioactivity was detected, including in Fractions 35 to 50 in which the degradation products of the carrier heparin were detected by the carbazole reaction. These results suggest that the [14C]fibronectin preparation did not contain glycosaminoglycans or proteoglycans that are sensitive to nitrous acid degradation such as heparin or heparan sulfate.

Heparin-binding Fragments—Our demonstration of high affinity glycosaminoglycan sites in fibronectin suggested that it might be possible to isolate proteolytic fragments still containing these sites. Cellular fibronectin was permitted to bind to heparin-agarose affinity columns, then digested in situ with α-chymotrypsin or pronase as described under "Experimental Procedures." The proteolytic fragments that were eluted from the column were compared to those that remained bound after extensive washing. Bound fragments were eluted with boiling 2% SDS; an SDS-polyacrylamide gel analysis is shown in Fig. 11.

After a brief digestion with chymotrypsin, several large fragments remained bound to the heparin affinity column, including a previously isolated (31), large fragment with an apparent molecular weight of 205,000 (Fig. 11b). Further digestion resulted in the generation of a previously isolated component of 160,000 daltons (31) thought to contain a collagen-binding site (Fig. 11c).

Pronase digestion of the 160,000-dalton fragment generates a fragment of 50,000 daltons (Fig. 11d); this fragment is relatively stable, since 2-fold increases or decreases in pronase concentrations or in digestion times had little effect on these results. We therefore identified this 50,000-dalton fragment as a protease-resistant, heparin-binding domain of fibronectin. This fragment has recently been recovered from heparin affinity columns using elution by high ionic strength, and the fragment will re-bind to heparin affinity columns with greater than 90% efficiency.

The material that did not bind to the column after chymotryptsin digestion included a major component of 40,000 daltons. This fragment will bind to gelatin (denatured collagen) affinity columns (Fig. 11e) and it co-migrates electrophoretically with a previously isolated chymotryptic fragment containing a collagen-binding site (26).

The material that did not bind to the heparin columns subjected to a subsequent pronase consisted primarily of a fragment of 90,000 daltons (Fig. 11g). If binding and digestion

K. M. Yamada and D. W. Kennedy, unpublished data.
Our major conclusions concerning the interactions of purified cellular fibronectin with glycosaminoglycans are: 1) fibronectin binds hyaluronic acid, heparin, and, to a lesser extent, heparan sulfate, but there is no evidence for binding of chondroitin sulfate or glycopeptides; 2) the binding of hyaluronate and heparin is saturable, of moderately high affinity, and at separate sites; 3) the binding of both macromolecules is not inhibited by EDTA, and occurs under physiological salt conditions; high salt inhibits heparin binding more than hyaluronic acid binding; and 4) heparin-binding domains of fibronectin can be isolated by affinity chromatography and proteolytic cleavage; one such domain of a molecular weight of 50,000 can be recovered per monomer of fibronectin.

Our results suggest that the binding of heparin and hyaluronic acid is specific and not due to simple nonspecific electrostatic binding of charged polymers. The binding site for heparin can be localized to a specific 50,000-dalton fragment of the molecule that is resistant to digestion by the broad-spectrum protease pronase. These findings suggest that the previously described domains of polypeptide structure in cellular fibronectin that were identified by spectrophotometric studies (32, 33) may actually represent specific binding site domains for the binding of various macromolecules.

Besides the heparin-binding fragment, another protease-resistant domain containing the collagen-binding site of fibronectin has been isolated following digestion by chymotrypsin, trypsin, or mast cell protease (26, 34, 35), or by pronase (Fig. 11). Since this 40,000-dalton collagen-binding fragment is separate from the heparin-binding fragment (Fig. 11), and since another previously described fragment appears to contain a cell-surface binding site (31), a speculative model of fibronectin binding sites is presented in Fig. 12 (see Ref. 31 for further discussion of other evidence from several laboratories). In this model, a number of adhesive or binding interactions of fibronectin can be explained by the presence of specific binding site domains in fibronectin that function to link together various other molecules. For example, fibronectin could mediate the attachment of certain glycosaminoglycans to the cell surface via the sites for these molecules and for a cell
Fibronectin-Glycosaminoglycan Interactions

In addition, detailed studies of the binding of hyaluronate by cultured cells suggest that cells contain high affinity receptors for hyaluronate (50, 51) as well as mechanisms for binding heparin and heparan sulfate (52). Hyaluronic acid binding is of particular interest, since this molecule may play roles in cellular adhesiveness (53) and in a variety of developmental morphogenetic events (54–56). It will therefore be important to determine whether this binding of glycosaminoglycans to cells is due to the high affinity binding sites of cell-surface fibronectin.

In addition, it is possible that the fibronectin found in extracellular spaces and in or near basement membranes might serve as a cross-linking, structural glycoprotein. Since it can bind to collagen types I, II, III, and IV, as well as to certain glycosaminoglycans (reviewed in Refs. 1 and 2), it may also play an important role in the internal organization of these matrices.

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Fig. 12. Model for functional domains of fibronectin. Three types of binding sites identified in fibronectin, and the sizes of the proteolytic fragments to which these sites have been localized, are indicated for various chymotryptic and pronase fragments of fibronectin. The order of the peptides has not been determined.

surface receptor. The presence of all of the indicated sites on each monomer has not been proven, although recent studies of several separate cell- or collagen-binding fragments provide results consistent with this notion (31). In addition, it is of interest that disulfide bonds and oligosaccharide chains have also been shown to be localized to specific regions or domains of the molecule (36, 37).

Glycosaminoglycans are also known to bind to collagen (reviewed in Ref. 38). The binding to collagen appears to be via simple electrostatic interactions. In contrast to our results with fibronectin, a variety of sulfated glycosaminoglycans bind effectively to collagen, whereas hyaluronic acid does not. We find that treatment with even very high concentrations of collagensase does not alter fibronectin binding to heparin or hyaluronic acid (Tables I and II).

Specific binding of hyaluronic acid by cartilage proteoglycans has been characterized by Hascall and others (20, 39, 40; see also 41). By chondroitinase ABC and nitrous acid degradation procedures, we do not find evidence for such proteoglycans in our fibronectin preparations; it is pertinent that ionic acid and N-acetylgalactosamine were also not found in our fibronectin (13). However, it has not been excluded that the binding might be related to that of the cartilage-type “link” protein; this possibility is currently under investigation.

It is curious that the binding of heparin and fibronectin shows high and lower affinity components (Fig. 9). It is possible that these two binding affinities reflect the presence of the two different classes of heparin discovered in studies of the anticoagulant activity of heparin (42).

Although fibronectin is a major constituent of cell surfaces, extracellular spaces, and possibly basement membranes (1, 2, 43), little is known about how it might interact with glycosaminoglycans in vivo. Culp and co-workers (44–46) have reported that adhesive regions on the undersurface of cultured cells are enriched in fibronectin and in heparan sulfate, chondroitin sulfate, and hyaluronic acid. Fibronectin exogenously added to cultured cells can be cross-linked to sulfated proteoglycans by chemical cross-linkers (12), suggesting that they may also bind in vivo. Glycosaminoglycans or proteoglycans present on fibroblastic cells are known to be released by proteases, but not by high concentrations of salts or EDTA, suggesting that there is a protease-sensitive component to their binding (47, 49). Some of this binding may be via noncovalent interactions, since heparan sulfate on the cell surface can be released from cells by incubating cultures with heparin (49).