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Binding of Fibronectin and Its Proteolytic Fragments to Glycosaminoglycans

EXPOSURE OF CRYPTIC GLYCOSAMINOGLYCAN-BINDING DOMAINS UPON LIMITED PROTEOLYSIS*

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Binding of intact plasma fibronectin and its proteolytic fragments to glycosaminoglycans immobilized on agarose beads was systematically compared at different ionic strengths. In low ionic strength buffer, intact fibronectin bound to heparin and high sulfated heparan sulfate, but not to low sulfated heparan sulfate, dermatan sulfate, chondroitin sulfates A and C, or hyaluronic acid. Fractionation of the thermolysin digest of fibronectin on the glycosaminoglycan-Sepharoses at low ionic strength revealed that three groups of fragments, *i.e.* M_r = 150,000-140,000, 24,000, and 16,000 (150K-140K, 24K, and 16K) fragments, were capable of binding to glycosaminoglycans with different specificities and affinities. The 150K-140K fragments exhibited the same specificities as intact fibronectin, binding only to heparin and high sulfated heparan sulfate. However, the 24K fragment bound not only to these two glycosaminoglycans but also to low sulfated heparan sulfate and other glycosaminoglycans as well. The 16K fragments were also capable of binding to most glycosaminoglycans with lower affinity than the 24K fragment. These results suggest that the binding sites in the 24K and 16K fragments are cryptic in the intact protein, but are exposed after limited proteolysis. The binding of fibronectin and its fragments to glycosaminoglycans is dependent on the ionic strength. At physiologic ionic strength, only heparin-Sepharose could bind intact fibronectin. Similarly, only heparin-Sepharose could bind the 150K-140K and 24K fragments, but not the 16K fragments, at the same ionic condition. Other glycosaminoglycan-Sepharoses did not retain significant amounts of any of the fibronectin fragments, suggesting that the affinity of plasma fibronectin and its fragments to heparan sulfate and other glycosaminoglycans, except heparin, is not strong enough to achieve stable mono- (or di-) valent binding under physiologic conditions.

Fibronectin is a multifunctional glycoprotein which is abundant in the extracellular matrices and in the basement membranes, as well as in plasma and other body fluids. It plays a role in cell-cell and cell-substrate adhesion, cellular motility and differentiation, wound healing, and reticuloendothelial clearance (1–10). These pleiotropic biological functions of fibronectin are primarily based on its ability to bind to a large variety of biomolecules, including collagens (or gelatin), heparin and some other GAGs,[1] fibrin, complement component C1q, actin, DNA, gangliosides, and other, more poorly characterized surface components of eukaryotic cells and certain bacteria (for recent reviews, see Refs. 6–10).

The pleiotropic functions of fibronectin are based on clear structural domains. Currently, four structural domains have been identified (8–10, 22–24). These domains can be obtained as 150K-140K, 43K[2], 24K, and 21K fragments upon mild thermolysin digestion (22–24).

Interaction of fibronectin with heparin and other GAGs is of particular interest because fibronectin and GAGs are the major components of the extracellular matrices and the basement membranes (*i.e.* lamina rara), and their interaction appears to be important for the organization of these structures (10–12). These components are also enriched in cell-substrate adhesion sites, suggesting the importance of their interaction in cellular adhesion (13). In addition, heparin-fibronectin interaction has been shown to be essential for the fibronectin-dependent phagocytosis by macrophages (5) and for the heparin-induced cryoprecipitation of plasma (3, 14).

Binding specificity of GAGs to fibronectin has been studied by several investigators using different methods (15–18). Heparin consistently binds to fibronectin, irrespective of the assay protocols. However, the binding of other GAGs is variable, depending on the methods and the source of fibronectin used. So far, HS (16–19), HA (15, 16, 19, 20), and chondroitinase-sensitive sulfated proteoglycans (21) have been reported to bind to, or closely associate with, fibronectin. In these previous studies, however, the experimental design has been inappropriate to compare the binding affinity of various domains of fibronectin to various GAGs (see "Discussion").

Recently, an improved method has been developed to couple high concentrations of GAGs to Sepharose (Refs. 27 and 28; see "Experimental Procedures"). With the use of such high density GAG-Sepharose columns, we could directly compare the binding activity of intact fibronectin and its proteolytic fragments to various types of GAGs. The results indicate that

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[1] The abbreviations used are: GAG, glycosaminoglycan; HS, heparan sulfate; DS, dermatan sulfate; CS-A, chondroitin sulfate A; CS-C, chondroitin sulfate C; HA, hyaluronic acid; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid. 24K fragment is used to indicate the fragment of M_r = 24,000, for example.

[2] The molecular weight of the fragment capable of binding to gelatin was tentatively determined to be 40,000 (22), but it has been recently re-estimated to be 43,000 (K. Sekiguchi and S. Hakomori, unpublished observation).

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(i) plasma fibronectin contains three distinct GAG-binding domains, *i.e.* the 150K-140K, 24K, and 16K fragments, which differ in their affinities and specificities to various GAGs; (ii) the binding sites in the 24K and 16K fragments appear to be cryptic in intact molecules; and, (iii) the affinities of intact fibronectin and its fragments to most GAGs, except heparin, are not strong enough to support mono- (or di-) valent binding at physiologic ionic strength. The biological implications of these observations are discussed.

EXPERIMENTAL PROCEDURES

Materials

Plasma fibronectin was purified from freshly drawn hamster blood as described previously (22). CS-A and CS-C were isolated from whale and shark cartilage, respectively. DS was prepared from chicken comb. HA was obtained from umbilical cord and partially degraded into large oligosaccharide fragments (average $M_r = 8000$) by testicular hyaluronidase. High sulfated HS containing 15.2% sulfate was prepared from rat kidney (29). Low sulfated HS containing 8.4% sulfate was obtained from porcine kidney (30). Heparin from porcine intestinal mucosa was purchased from Wako Chemical (Tokyo, Japan). Gelatin-Sepharose (5 mg of gelatin/ml of packed gel) and fibrin-Sepharose (5 mg of fibrin/ml of packed gel) were prepared as described previously (23, 24). Heparin-agarose was purchased from Pierce (Rockford, IL). Ampholines, pH 3.5–10, 7–10, and 4–6, were obtained from LKB (Gaithersburg, MD).

Methods

Preparation of GAG-Sepharose—GAGs were immobilized on amino-Sepharose with water-soluble carbodiimide according to Funahashi *et al.* (28). Briefly, Sepharose 4B (Pharmacia) was converted to epoxy-activated Sepharose with epichlorohydrin and then to amino-Sepharose with concentrated ammonia. The amino-Sepharose thus prepared contained approximately 45 μmol of amino group/g of wet gel (28). GAGs were coupled to the amino-Sepharose by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide or by reductive amination with $NaCNBH_3$. The GAG content (milligrams/g of wet gel) of these derivatized Sepharoses is: heparin-Sepharose, 18.6; high sulfated HS-Sepharose, 13.2; low sulfated HS-Sepharose, 29.1; DS-Sepharose, 18.4; CS-A-Sepharose, 27.5; CS-C-Sepharose, 22.1; HA-Sepharose, 10.7.

Purification of 150K-140K, 24K, and 16K Thermolysin Fragments—Intact hamster plasma fibronectin (1.2 mg/ml) was digested with thermolysin (2.5 μg/ml) at 22 °C for 4 h. The digestion was terminated by adding 0.2 M EDTA to give a final 5 mM EDTA. The digest was sequentially passed through gelatin-Sepharose and DEAE-cellulose as described previously (23). The DEAE-cellulose column was washed with 25 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA and 80 mM NaCl (Tris/NaCl (80mM)) to elute the 21K fragment, and then washed with Tris/NaCl(200 mM) to elute the 150K-140K fragments (23). The DEAE-cellulose-unbound fraction, which contained both the 24K and the 16K fragments, was further sequentially fractionated on fibrin-Sepharose (bed volume, 20 ml) and heparin-agarose (Pierce; bed volume, 20 ml). The 24K fragment was eluted from the fibrin-Sepharose with Tris/NaCl(50 mM) containing 4 M urea. After dialysis against Tris/NaCl(50 mM), the 24K fragment was further purified on heparin-agarose (Pierce; bed volume, 20 ml). The 16K fragments, which bound to the heparin-agarose, were eluted with Tris/NaCl(1 M). All purified fragments were finally dialyzed against Tris/NaCl(50 mM). The purity of the fragments used in the present experiments is shown in Fig. 1.

GAG-binding of Intact Fibronectin and Its Fragments—Each GAG-Sepharose was packed in a small column (bed volume, approximately 0.2 ml) and prewashed with Tris/NaCl(1 M) and then Tris/NaCl(50 mM) unless otherwise indicated. Intact fibronectin or its thermolysin fragments (0.16–0.87 mg), dissolved in 0.5 ml of Tris/NaCl(50 mM), was applied to the column at a flow rate of 50 μl/min. The column was washed with no less than 7 ml of Tris/NaCl(50 mM) at the same flow rate. The bound proteins were eluted with Tris/NaCl(1 M). Fractions of 1.4 ml were collected and assayed for protein by absorbance at 280 nm. In some experiments, the Tris/NaCl(50 mM) buffer was replaced by Tris/NaCl(135 mM) to study the binding at physiologic ionic strength.

Other Procedures—SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (31) with 9.5% polyacrylamide gels. Samples were reduced with 2% (v/v) 2-mercaptoethanol. The following proteins were used as molecular weight standards: skeletal muscle myosin (200,000), β-galactosidase (116,000), phosphorylase *b* (94,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Isoelectric focusing was performed in 6% polyacrylamide gels using Ampholines, pH 3.5–10, 4–6, and 7–10. Proteins were determined by the method of Lowry *et al.* (32) with bovine serum albumin as a standard.

RESULTS

Binding of Intact Fibronectin to GAG-Sepharoses—Binding of intact fibronectin to various types of GAGs was systematically compared by affinity chromatography on GAG-Sepharoses at different ionic strength (Table I). At low ionic strength, intact fibronectin bound to Sepharose columns containing heparin and high sulfated HS (sulfate content, 15.2%) almost quantitatively, but it did not bind to other Sepharose columns containing low sulfated HS (sulfate content, 8.4%), DS, CS-A, CS-C, or HA. The sulfate content critically affects the binding of intact fibronectin to HS-Sepharoses under this condition. In contrast, fibronectin could not bind to any HS-Sepharose, irrespective of sulfate content, at physiologic ionic strength. Even heparin-Sepharose failed to retain nearly 20%

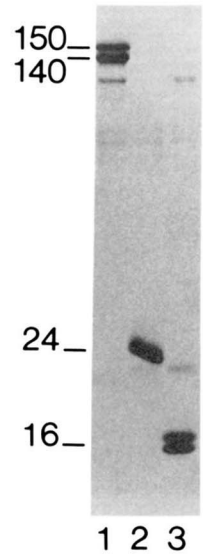

FIG. 1. **SDS-polyacrylamide gel electrophoresis of the purified thermolysin fragments.** *Lane 1*, the 150K-140K fragments (10 μg); *lane 2*, the 24K fragment (5 μg); *lane 3*, the 16K fragments (5 μg).

TABLE I

Binding of intact fibronectin to GAG-Sepharoses

Binding of intact fibronectin (390 μg) to GAG-Sepharoses was examined in low salt (Tris/NaCl(50 mM)) and physiologic salt (Tris/NaCl(135 mM)) buffer solutions as described under "Experimental Procedures."

GAG	Protein bound	
	Tris/NaCl (50 mM)	Tris/NaCl (135 mM)
	%	
Heparin	96	79
High sulfated HS	95	7
Low sulfated HS	5	10
DS	4	9
CS-A	6	5
CS-C	8	6
HA	4	3

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of input protein under the same conditions (Table I). Sepharose-linked DS, CS-A, CS-C, or HA did not bind intact fibronectin either. These results indicate that the affinities of HS and other GAGs, except heparin, to intact fibronectin are rather weak at physiologic conditions and cannot stabilize mono- (or di-) valent interaction between GAGs and fibronectin (see also "Discussion").

Binding of Thermolysin Fragments to GAG-Sepharoses—Binding of fibronectin to GAG-Sepharoses was also examined after thermolysin digestion (Table II). At low ionic strength, approximately 60% of the protein in the thermolysin digest was adsorbed on heparin- and high sulfated HS-Sepharoses. Interestingly, significantly increased amounts of protein fragments, as compared with intact protein (see Table I), were adsorbed on low sulfated HS-, DS-, and CS-A-Sepharoses. At physiologic ionic strength, heparin-Sepharose retained 43% of the total input protein, which is significantly less than that retained at low ionic strength. Other GAG-Sepharoses, including high sulfated HS-Sepharose, did not adsorb a significant amount of the protein fragments (Table II), being consistent with the binding of intact fibronectin to these GAG-Sepharoses at the same ionic strength.

TABLE II

Binding of the thermolysin digest of fibronectin to GAG-Sepharoses

Intact fibronectin (0.89 mg/ml) was digested by thermolysin (2.5 μg/ml) in Tris/NaCl(50 mM) or Tris/NaCl(135 mM) containing 2.5 mM $CaCl_2$ at 22 °C for 4 h. The digestion was terminated by adding final 5 mM EDTA. The digest (875 μg) was fractionated on GAG-Sepharoses as described under "Experimental Procedures."

GAG	Protein bound	
	Tris/NaCl (50 mM)	Tris/NaCl (135 mM)
	%	
Heparin	65	43
High sulfated HS	59	3
Low sulfated HS	21	6
DS	16	7
CS-A	13	2
CS-C	8	6
HA	7	6

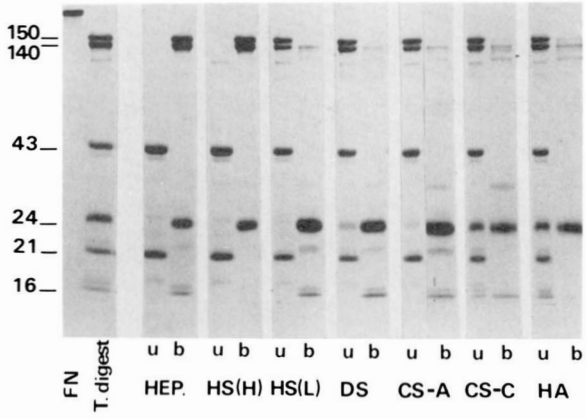

FIG. 2. **SDS-polyacrylamide gel electrophoresis of the fragments bound to GAG-Sepharoses at low ionic strength.** The thermolysin digest was prepared and fractionated on various GAG-Sepharoses in Tris/NaCl(50 mM) buffer as described in Table II. The unbound (*u*) and bound (*b*) fragments were analyzed by SDS-polyacrylamide gel electrophoresis. The bound fragments were dialyzed against 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA and 50 mM NaCl before analyzing on the gel. Ten micrograms of protein were loaded on each lane except intact fibronectin (*FN*, 5 μg) and the whole thermolysin digest (*T. digest*, 15 μg). *HEP.*, *HS(H)*, and *HS(L)*, heparin, high sulfated HS, and low sulfated HS, respectively.

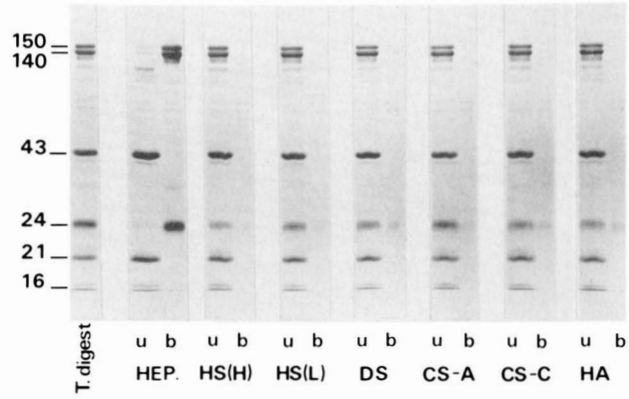

FIG. 3. **SDS-polyacrylamide gel electrophoresis of the unbound (*u*) and bound (*b*) fragments on GAG-Sepharoses at physiologic ionic strength.** The thermolysin digest was prepared and fractionated on GAG-Sepharoses in Tris/NaCl(135 mM) buffer as described in Table II. The protein amounts loaded on each lane are: the whole thermolysin digest (*T. digest*), 15 μg; heparin (*HEP.*)-unbound fragments, 15 μg; heparin-bound fragments, 12.5 μg; high sulfated HS (*HS(H)*)-unbound fragments, 15 μg; high sulfated HS-bound fragments, 0.5 μg; low sulfated HS (*HS(L)*)-unbound fragments, 15 μg; low sulfated HS-bound fragments, 0.8 μg; DS-unbound fragments, 15 μg; DS-bound fragments, 1.0 μg; CS-A-unbound fragments, 15 μg; CS-A-bound fragments, 0.4 μg; CS-C-unbound fragments, 15 μg; CS-C-bound fragments, 0.9 μg; HA-unbound fragments, 15 μg; HA-bound fragments, 0.9 μg. The relative protein amounts loaded for the unbound and bound fragments are adjusted to reflect the relative protein recoveries of the two fragments.

Fragments which bound and did not bind to GAG-Sepharoses at low ionic strength were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). Heparin-Sepharose bound the 150K-140K and 24K fragments, but failed to bind the 43K and 21K fragments, as previously reported (22, 24). In addition, two fragments which migrated approximately at the M_r = 16,000 region were also adsorbed to heparin. These fragments are collectively referred to as "16K fragments" in this paper. Thus, three sets of fragments, 150K-140K, 24K, and 16K fragments, were capable of binding to heparin at low ionic strength. Similarly, both the 150K-140K and 24K fragments also bound to high sulfated HS-Sepharose. The 16K fragments, however, were not detected in either bound or unbound fractions of the same column, but were found in the fraction eluted from the column upon extensive washing with Tris/NaCl(50 mM) buffer, *i.e.* the 16K fragments were loosely adsorbed on the column. In fact, a separate experiment with the purified 16K fragments indicated that the fragments were initially retained on high sulfated HS-Sepharose, but increasing amounts of the fragments were eluted in the later fractions upon prolonged washing with Tris/NaCl(50 mM) (see below and Fig. 6).

Unexpectedly, the 24K fragment was also capable of binding to Sepharose columns containing low sulfated HS, DS, and CS-A, and to columns containing CS-C and HA to a lesser extent. Similarly, the 16K fragments could bind to low sulfated HS, DS, CS-A, and CS-C. However, they were barely detected in the bound fractions of HA-Sepharose. In contrast, the 150K-140K fragments did not bind significantly to low sulfated HS, DS, CS-A, CS-C, or HA, to which intact fibronectin did not bind either. The binding of the 24K and 16K fragments to Sepharose-linked low sulfated HS, DS, and CS-A may account for the increased binding of fibronectin after thermolysin digestion (Table II).

Under physiologic ionic conditions, however, none of the thermolysin fragments were retained on GAG-Sepharoses except heparin-Sepharose (Fig. 3). Heparin-Sepharose ad-

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sorbed most of the 150K-140K and 24K fragments at physiologic ionic strength, but it failed to bind the 16K fragments. The 24K and 16K fragments, which bound to most of the GAG-Sepharoses at low ionic strength, were not retained on the GAG-Sepharoses at physiologic ionic strength. Only a trace of the 24K fragment was detectable in the bound fractions of the Sepharose columns, suggesting that the affinity of the 24K fragment to most of GAGs in a physiologic ionic environment is not strong enough to stabilize monovalent interaction between the fragment and the GAGs.

Binding of Purified Fragments to GAG-Sepharoses at Low Ionic Strength—The distinct specificities of the three GAG-binding fragments at low ionic strength were further examined with purified fragments. As shown in Fig. 4, the 150K-140K fragments were quantitatively retained on heparin- and high sulfated HS-Sepharose columns, whereas the columns containing other GAGs, including low sulfated HS, retained less than 18% of the fragments. However, it is notable that the elution profiles of the 150K-140K fragments from the columns containing low sulfated HS, DS, CS-A, and CS-C exhibited significant retardation, indicating the presence of weak interaction between the 150K-140K fragments and these GAGs. No significant retardation was observed for the HA-Sepharose column.

In contrast, the 24K fragment could bind to virtually any type of GAG (Fig. 5). It was quantitatively retained by heparin-, high sulfated HS-, and low sulfated HS-Sepharoses. A majority of the fragment was also retained on DS-, CS-A-, and CS-C-Sepharoses. Even HA-Sepharose could retain 42% of the fragment loaded to the column. Binding of the 24K fragment to GAG-Sepharoses is not due to nonspecific adsorption, because this fragment does not bind to bovine serum albumin-Sepharose (data not shown) under the same conditions.

The 16K fragments could also interact with all of the GAG-Sepharoses with variable affinities (Fig. 6). The fragments were retained almost quantitatively on heparin-Sepharose. The fragments also bound to the columns containing high sulfated and low sulfated HS, DS, CS-A, and CS-C, although their binding was not stable; the fragments eventually began to come off the columns upon prolonged washing. Nevertheless, a considerable amount of the fragments (*i.e.* 34–57% of the input protein) was retained on these GAG-Sepharose columns even after washing with more than 50 column volumes of Tris/NaCl(50 mM). The failure to detect the 16K fragments in the bound fraction from the high sulfated HS-Sepharose chromatography of the thermolysin digest (Fig. 2) is considered to be due to the extensive washing with Tris/NaCl(50 mM), which eventually resulted in the removal of most of the bound fragments. The HA-Sepharose column retained 28% of the loaded fragments, but leakage of the fragments upon prolonged washing was not evident. These results are consistent with the results of the GAG-binding of the thermolysin digest and also indicate that the 16K fragments could bind to various types of GAGs *per se*, but not through association with the 24K fragment.

Stepwise Elution of the GAG-binding Fragments at Different Ionic Strengths—The GAG-binding properties of the thermolysin fragments at low and physiologic ionic strength were

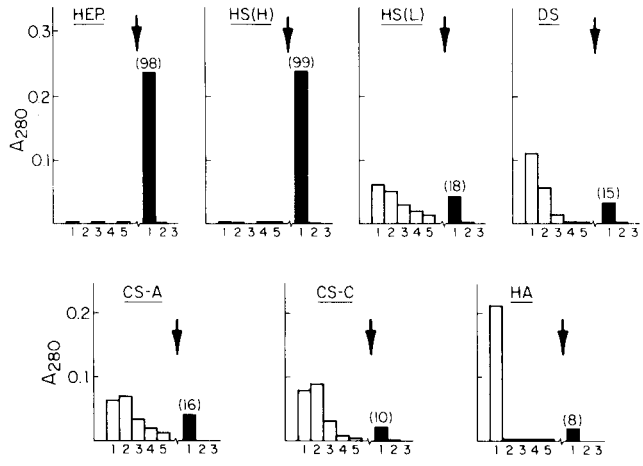

FIG. 4. **Binding of the 150K-140K fragments to GAG-Sepharoses.** The purified 150K-140K fragments (480 μg) in 0.5 ml of Tris/NaCl(50 mM) were applied to each GAG-Sepharose column. The chromatography on GAG-Sepharoses was performed as described under "Experimental Procedures." Five fractions were collected for unbound fragments (*open bars*) and three fractions were collected for bound fragments (*solid bars*). The *numbers* in *parentheses* show the recovery of the fragments in the bound fractions. Abbreviations are as in Fig. 2.

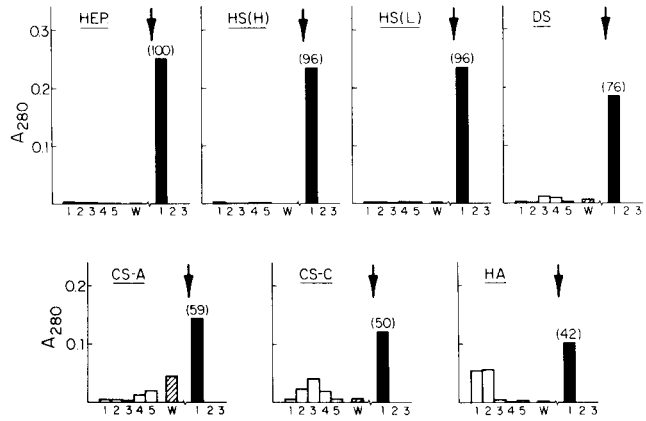

FIG. 5. **Binding of the 24K fragment to GAG-Sepharoses.** The purified 24K fragment (265 μg) in 0.5 ml Tris/NaCl(50 mM) was applied to each GAG-Sepharose column. The chromatography was performed as described in Fig. 2. except that the eluates (3–4 ml) after the first five fractions were pooled in a test tube and assayed for protein by absorbance at 280 nm. The amount of protein in this fraction, shown as a *hatched bar* (*W*), was normalized as the absorbance at 280 nm/1.4 ml. The *numbers* in *parentheses* show the recovery of the fragment in the bound fractions. Abbreviations are as in Fig. 2.

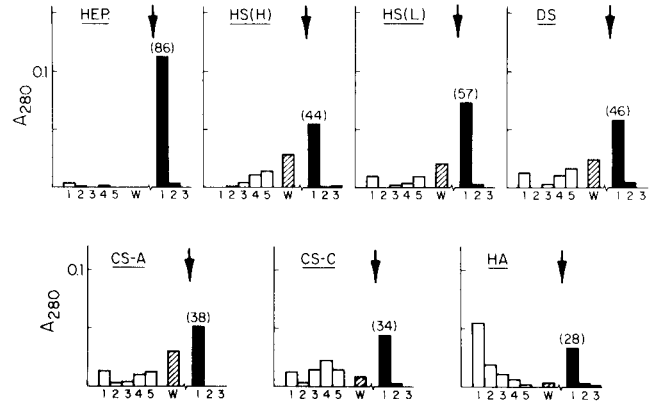

FIG. 6. **Binding of the 16K fragments to GAG-Sepharoses.** The purified 16K fragments (160 μg) in 0.5 ml of Tris/NaCl(50 mM) were applied to each GAG-Sepharose column. The chromatography was performed as described in Fig. 4. After collecting the first five fractions, the eluates (2.5–3.5 ml) were pooled and assayed for protein. The amount of protein in this fraction, shown as a *hatched bar* (*W*), was normalized as the absorbance at 280 nm/1.4 ml. The *numbers* in *parentheses* show the recovery of the fragments in the bound fractions. Abbreviations are as in Fig. 2.

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TABLE III

Stepwise elution of the fibronetin fragments preadsorbed on GAG-Sepharoses at low ionic strength

Intact fibronectin (0.95 mg/ml) was digested by thermolysin (2.5 μg/ml) in Tris/NaCl(50 mM) buffer as described in Table II. The digest (950 μg) was chromatographed on GAG-Sepharose columns equilibrated with Tris/NaCl(50 mM) as described under "Experimental Procedures." The columns were first washed with 28 column volumes of Tris/NaCl(50 mM), followed by sequential elution with 21 column volumes of Tris/NaCl(135 mM) and Tris/NaCl(1 M).

GAG	Protein recovered		
	Tris/NaCl (50 mM)	Tris/NaCl (135 mM)	Tris/NaCl (1 M)
		%	
Heparin	37	17	39
High sulfated HS	39	52	2
Low sulfated HS	66	21	4
DS	75	14	4
CS-A	74	18	2
CS-C	76	12	4
HA	74	11	4

further studied by stepwise elution of the fragments preadsorbed on various GAG-Sepharoses at low ionic strength. As shown in Table III, almost all the fragments adsorbed in low salt buffer were eluted by increasing the NaCl concentration to 0.135 M. Only heparin-Sepharose retained nearly 40% of the input fragments even after the elution with physiologic salt buffer, being consistent with the results shown in Table II.

The profiles of fragments eluted from GAG-Sepharoses (except heparin-Sepharose) at physiologic salt concentration were essentially identical with those of the fragments adsorbed at low ionic strength and eluted with high salt buffer (see Fig. 2; data not shown). The fragments eluted from heparin-Sepharose with physiologic salt buffer consist of the 150K-140K, 24K, and 16K fragments, whereas the fragments eluted by high salt buffer consist of the 150K-140K and 24K fragments (data not shown), also being consistent with the results shown in Figs. 2 and 3.

The affinity of the three GAG-binding fragments to heparin was further studied by large scale chromatography of the thermolysin digest on a heparin-agarose column. As shown in Fig. 7, stepwise elution with increasing salt concentrations separated the whole digest into five peaks. Peak I, which did not bind to heparin even at low ionic strength, contained the 43K and 21K fragments (Fig. 8, *lane 3*). Peaks II–IV were sequentially eluted from the column at physiologic salt concentration. Peak II contained predominantly the 16K fragments (Fig. 8, *lane 4*).[3] Peak III, which followed peak II with slight retardation, contained the 24K fragment (Fig. 8, *lane 5*). Peak IV, which came off the column as a broad peak after prolonged elution, contained the 150K-140K fragments (Fig. 8, *lane 6*). These fragments were eluted in a sharper peak with a buffer containing 0.2 M NaCl (Peak V; Fig. 8, *lane 7*). Thus, the affinities of the three GAG-binding fragments to heparin are considered to be in the following order: 150K-140K > 24K > 16K. This is in striking contrast to the binding of these fragments to "low affinity" GAGs (*i.e.* low sulfated HS, DS, CS-A, CS-C, and HA). The 24K fragment was capable of binding to low affinity GAGs most strongly, whereas the 16K fragments bound to these GAGs to a moderate extent, and the 150K-140K fragments could not bind to these GAGs.

[3] In this particular experiment, the thermolysin digest contained more of the upper band of the 16K fragments than the lower band because the fibronectin was digested with thermolysin for only 2 h (see also "Discussion").

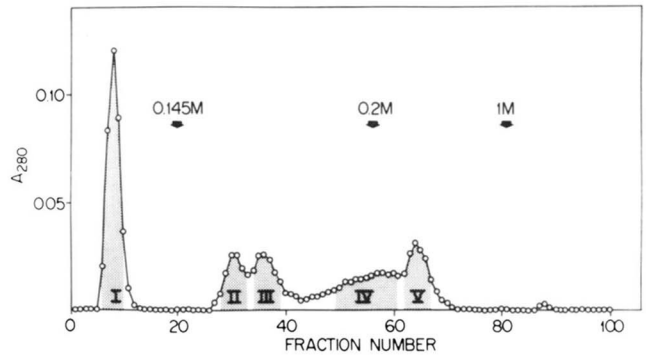

FIG. 7. **Heparin-agarose chromatography of the thermolysin digest.** Intact fibronectin (1.4 mg/ml) dissolved in 10 mM Tris-HCl (pH 7.6) containing 0.5 mM EDTA, 2.5 mM $CaCl_2$, and 0.05 M NaCl was digested by thermolysin (2.5 μg/ml) at 22 °C for 2 h. After inactivation of the protease by addition of 5 mM EDTA, the digest was applied to a heparin-agarose column (2.2 × 5.5 cm) which was equilibrated with the 10 mM Tris buffer containing 0.5 mM EDTA and 0.05 M NaCl. The column was extensively washed with the same buffer, and then eluted sequentially with the 10 mM Tris buffer containing 0.145, 0.2, and 1 M NaCl, in a stepwise manner. Fractions of 3 ml were collected. Each of the peak fractions, numbered *I* to *V*, was separately pooled and analyzed by SDS-polyacrylamide gel electrophoresis (see Fig. 8).

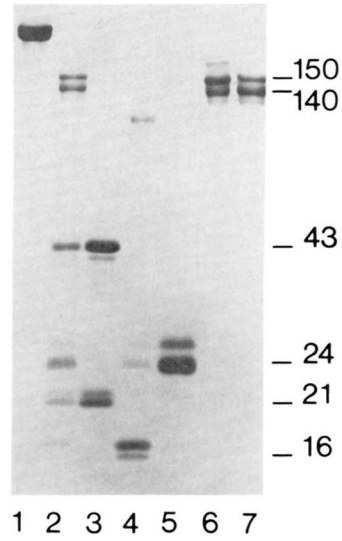

FIG. 8. **SDS-polyacrylamide gel electrophoresis of the fragments recovered in peaks I–V.** *Lane 1*, intact fibronectin (10 μg); *lane 2*, the thermolysin digest (10 μg); *lane 3*, peak I (10 μg); *lane 4*, peak II (10 μg); *lane 5*, peak III (10 μg); *lane 6*, peak IV (10 μg); *lane 7*, peak V (10 μg).

Isoelectric Points of GAG-binding Fragments—Since the binding of fibronectin and its fragments to GAGs is predominantly due to electrostatic interaction, the GAG-binding fragments are expected to be positively charged at neutral pH. Table IV shows the isoelectric points (pI values) of isolated thermolysin fragments determined by isoelectric focusing. The GAG-binding fragments, the 24K and 16K, have high pI values, although the 43K and 21K fragments, which do not bind to heparin, have low pI values. Other GAG-binding fragments, the 150K-140K fragments, have a rather low pI, but this is probably because the basic moieties in the GAG-binding region are neutralized by acidic moieties in other regions within the same fragments. Actually, the heparin-binding subdomain in the 150K-140K fragments was isolated

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TABLE IV
Isoelectric points of purified thermolysin fragments

Fragments	pI
150K-140K	5.10–5.25
43K	4.90–5.00
24K	8.40–8.60
21K	5.10–5.35
16K	8.50–9.00

as M_r = 35,000–37,000 fragments by Richter and Hörmann (45), and their pI was determined to be 8.2–8.9. Thus, all GAG-binding domains should be positively charged at neutral pH and can bind to negatively charged GAGs by electrostatic interaction.

DISCUSSION

The interaction of GAGs with fibronectin has been studied by several investigators using different methods (15–18). In these previous studies, interactions of HS, DS, CS-A, CS-C, and HA with fibronectin were based on the retention of GAGs on a fibronectin column (17, 18), co-precipitation of GAGs with cellular fibronectin at neutral pH (15), or agglutination of fibronectin-coated latex beads with GAGs (16). These methods, however, are technically inappropriate to compare the degree of interaction among the various domains of fibronectin and GAGs, because it is difficult to control the quantity of fibronectin fragments linked to the matrix. The reactivity of fibronectin and its fragments linked to the matrix depends on the steric locus of the linkages and the protein conformation as affected by coupling to agarose beads.

Recently, we have developed an improved method to immobilize GAGs to agarose beads with very high yields (27, 28). The procedure described in this paper, using such high density GAG-Sepharose columns, is novel in that the interactions of defined GAGs with various fibronectin domains can be compared. Since the quantity of GAGs bound to Sepharose is high and in approximately the same concentration range irrespective of the type of GAG, a direct comparison of the interaction of a defined fibronectin domain with various GAGs can be made. Such a comparison was impossible in previous studies.

Only through this new procedure is it possible to detect the ability of the 24K and 16K fragments, which are released by thermolysin digestion of fibronectin, to interact with "low affinity" GAGs, *i.e.*, low sulfated HS, DS, CS-A, CS-C, and HA at low ionic strength. Since intact fibronectin does not interact with these low affinity GAGs at either low or physiologic ionic strength, the binding activities of the 24K and 16K fragments to the low affinity GAGs must be cryptic in the intact protein.

The results of this and previous studies clearly indicate that fibronectin binds only to heparin at both physiologic and low ionic strengths. The binding of high sulfated HS to fibronectin is marginal and depends on pH and ionic strength. However, the binding of low sulfated HS and other low affinity GAGs is only possible at low ionic strength. The binding of HS to fibronectin-Sepharose was originally demonstrated by Laterra and Culp (17, 19) at low ionic strength. More recently, Stamatoglou and Keller (18) reported that HS binds to fibronectin-Sepharose under physiologic conditions, but that its binding is dependent on pH. Approximately 40% of HS failed to bind to fibronectin-Sepharose at pH 7.5 in a physiologic salt buffer solution (18), indicating that the interaction between HS and intact fibronectin is weak.

The novel binding activity of the 24K and 16K fragments with HS and other low affinity GAGs was only observed at low ionic strength. However, this does not necessarily preclude the physiologic significance of the interaction between these domains and low affinity GAGs. A weak interaction that occurs at physiologic ionic strength and pH may not be detectable by the solid phase chromatographic procedure because it involves extensive washing; however, only a solid phase chromatographic procedure can estimate and compare the degree of interaction in a quantitative manner. A weak interaction that is detectable at nonphysiologic, low ionic strength may well contribute to the organization of molecules in a biological system.

We reported previously that fibronectin consists of at least four structural domains which are obtained as 150K-140K, 43K, 24K, and 21K fragments upon mild thermolysin digestion (23, 24). These domains are referred to as "*Cell/Hep-2*," "*Gel*," "*Hep-1/Fib-1*," and "*Fib-2*" domains, respectively, according to their biological functions (24). Among these, the 150K-140K and 24K fragments were shown to bind to heparin (22, 24). In the course of the present investigation, we noticed the presence of another set of heparin-binding fragments, the 16K fragments. The fragments were consistently detected in every thermolysin digest, but they often migrated at the dye front on 9.5% acrylamide gels (see also Ref. 23). The fragments appear to correspond to 16K fragments that were generated from a 56K fragment, a precursor to the 43K *Gel* domain, upon prolonged thermolysin digestion (23). Thus, the 16K fragments may represent another constitutive domain which can be referred to as the "*Hep-3*" domain. This domain is considered to be located between the *Gel* domain and the *Cell/Hep-2* domain because the *Gel* domain must be adjacent to the NH_2-terminal *Hep-1/Fib-1* domain (25, 33, 34).

The presence of a cryptic GAG-binding domain suggests that intact fibronectin is folded in such a conformation that these cryptic sites are associated with complementary, negatively charged region(s) within the same molecule. In support of this, Hörmann recently proposed a model in which monomeric molecules are assumed to be folded in such a way that alternately charged domains in the NH_2-terminal region associate with the complementary charged COOH-terminal region within the same molecule (46). Isoelectric points of the purified domain fragments and their alignment in the intact subunit chains also support this model. Similar intramolecular ionic association was also proposed by Williams *et al.* (39) from the viscosity measurement of a fibronectin solution in different ionic environments.

It is tempting to speculate that fibronectin may be unfolded in the extracellular matrix. In support of this, binding of collagen has been shown to induce unfolding of fibronectin (39). Since fibronectin contains two latent GAG-binding sites capable of binding to HS and other low affinity GAGs, these GAGs may also interact with unfolded fibronectin in the extracellular matrix. Although the affinities of these GAGs to each binding domain are not strong enough to support monovalent binding under physiologic conditions, multiple interactions between these GAGs and polymerized fibronectin in the matrix may stabilize their interactions. In fact, Perkins *et al.* (21) demonstrated that chondroitinase-sensitive sulfated proteoglycans were cross-linked to fibronectin on the fibroblast surface. Inefficient incorporation of soluble fibronectin into the extracellular matrix (42–44) could be due to its folded configuration.

Our data also suggest that the three GAG-binding fragments are different in their specificities and affinities to various types of GAGs. At low ionic strength, the 150K-140K fragments could not bind to low sulfated HS, DS, CS-A, CS-C, or HA, to which the 24K and 16K fragments could bind.

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However, the 150K-140K fragments could bind to heparin with higher affinity than the 24K and 16K fragments. Even between the latter fragments, the 24K fragment could bind to these low affinity GAGs more strongly than the 16K fragments. These results are unique as compared to previous studies by other investigators who only studied the interactions of fragments with heparin. Richter *et al.* (25) showed that the cathepsin D digest of human plasma fibronectin was separated into four groups of fragments upon heparin-Sepharose chromatography with increasing salt concentration. Elution of a fragment with M_r = 140,000, which appears to contain the same GAG-binding site as the 150K-140K fragments, required the highest salt concentration (*i.e.* 0.25–0.5 M); a M_r = 70,000 fragment, which appears to contain the NH_2-terminal *Hep-1/Fib-1* domain, was eluted with 0.1–0.25 M NaCl (25), being consistent with our results. Hayashi and Yamada (26) showed that there were three groups of heparin-binding fragments whose binding was differently affected by divalent cations. A differential affinity of the three GAG-binding domains to various GAGs, and the presence of a cryptic binding locus in fibronectin, neither of which have been apparent in previous studies, are clearly demonstrated in this paper. A possible modulation of the binding activity through a folding/unfolding mechanism will undoubtedly be an important topic in future fibronectin research.

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Binding of fibronectin and its proteolytic fragments to glycosaminoglycans. Exposure of cryptic glycosaminoglycan-binding domains upon limited proteolysis.

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