Fibronectin Binding to Glomerular Basement Membrane is Altered in Diabetes

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SUMMARY

We examined the ability of fibronectin, an extracellular glycoprotein that interacts with cell surfaces and matrix components, to bind to glomerular basement membrane and the effect of diabetes on this binding. ^{125}I-labeled fibronectin binding to rat glomerular basement membrane (GBM) was dose dependent, related to time and amount of basement membrane, and inhibited by unlabeled fibronectin but not by unrelated proteins. Binding was reduced ~60% when GBM was pretreated with collagenase and ~24% when pretreated with chondroitinase plus heparinase. Treatment with NaCl had little effect on binding, whereas reduction with β-mercaptoethanol removed ~25% of the bound ^{125}I-fibronectin. Binding to samples prepared from rats with streptozocin-induced diabetes was significantly increased compared with that observed with control preparations at all concentrations of fibronectin and of basement membrane tested. The findings provide direct evidence that fibronectin binds to GBM and that this binding, which represents a biologic function of the protein, is enhanced in diabetes. *Diabetes* 36:758–63, 1987

Fibronectin is one of several extracellular glycoproteins that interact with cell surfaces and with each other to influence cell growth, development, and adhesion and to modulate biologic properties of extracellular matrices (1). It is present in large amounts in plasma as a soluble protein and in an insoluble form at cell surfaces. The two forms are similar but not identical and contain subunits of ~220,000 M_r that are joined by disulfide bonds into dimers and multimers (2–5). Fibronectin is synthesized by various cells, including fibroblasts and endothelial cells (6–9), and has been demonstrated by immunofluorescent techniques in the connective tissue matrix (10,11) and in basement membranes (11–13). In the renal glomerulus, fibronectin has been localized to the mesangial matrix (12,14–18), and hence the protein's presence is considered a marker for glomerular mesangium in renal biopsy specimens (19). It is not clear, however, whether the fibronectin that is faintly detected immunohistochemically along the subendothelial part of the glomerular basement membrane (GBM) and in the lamina rara interna (12,14,20) represents an integral component that derives from local production (8,12,14) or whether its presence in the peripheral GBM derives from entrapment of circulating fibronectin by the glomerular filtration barrier (16). The latter is suggested by the finding that type IV collagen, which is the major collagen type in GBM, purified from EHS sarcoma inhibits the binding of fibronectin to ^{125}I-labeled type I collagen (21), although in solution it does not bind fibronectin (22). Furthermore, Courtoy and Boyles (23) reported that fibronectin is only faintly visible in the microvascular basement membrane of extensively perfused preparations but is concentrated in and localized to the mesangial-endothelium interstitia. Regardless of its origin, it is believed that fibronectin exerts ligand binding and biologic properties in basement membranes that are similar to those that it intrinsically possesses and exhibits in other tissue sites (12) and thus could influence organizational features of this extracellular matrix. Indeed, the results of a recent study directly suggest a role for fibronectin in glomerular cell adhesion (24). We therefore examined the ability of fibronectin to bind to purified GBM. Because diabetes alters organizational and functional features of GBM, we also studied the effect of diabetes on this binding. Furthermore, because nonenzymatic glycosylation of fibronectin, a process that is enhanced in diabetes, has been shown to alter the protein's interaction with matrix macromolecules (25,26), examination of the influence of in vivo exposure to a hyperglycemic milieu on the ability of basement membrane to bind native fibronectin was considered of interest.

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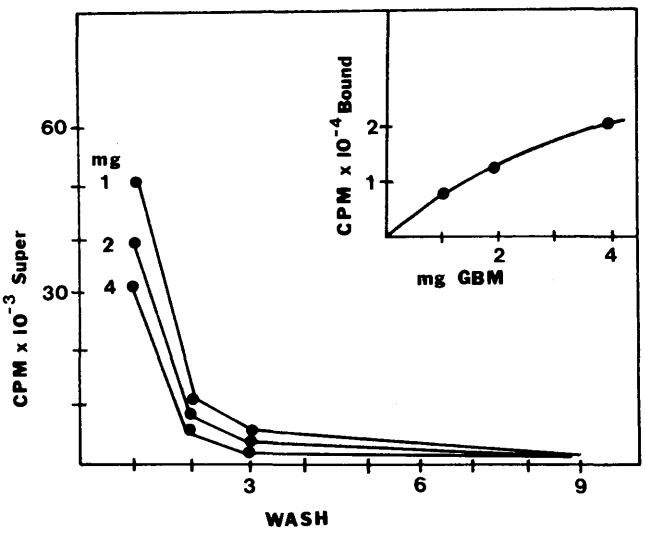

FIG. 1. Binding of ^{125}I-labeled fibronectin to glomerular basement membrane (GBM). In each series of incubations, indicated amounts of fibronectin and GBM were incubated with 90,000 cpm of radiolabeled fibronectin in 0.6 ml PBS containing 0.1% albumin for 3 h at room temperature. After incubation, basement membranes were collected by rapid centrifugation and repeatedly washed with 1 ml PBS containing 0.1% BSA. Radioactivity was counted in each supernatant and in final basement membrane pellet (*inset*). Data are representative of 4 separate experiments.

MATERIALS AND METHODS

Fibronectin was purified from human plasma by described techniques (27) or was purchased commercially (Cooper Biomedical, Malvern, PA). ^{125}I-fibronectin (sp act 1.43–2.51 μCi/μg) was obtained from New England Nuclear (Boston, MA). The purity of all preparations was confirmed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography, which demonstrated the characteristic 220,000-M_r components on reduction.

Basement membrane was purified from glomeruli isolated from age-matched control and diabetic rats according to described techniques (28,29). Treatment with DNase was routinely employed during the purification procedure to ensure that the preparations were free of DNA (28). Diabetic animals were killed 4 wk after the induction of diabetes with an injection of 65 mg/kg i.v. streptozocin and weighed 120–150 g when injected. At death, body weights were significantly lower and blood glucose concentrations were significantly higher in diabetic rats compared with controls (240 ± 12 vs. 298 ± 6 g and 20.0 ± 1.8 vs. 7.2 ± 0.8 mM, respectively). Glomeruli from four to six animals in each group were pooled for individual preparations of basement membranes. Six separate preparations of GBM were used for experiments comparing fibronectin binding by control and diabetic GBM.

Binding of fibronectin was examined by incubating purified rat GBM with the radiolabeled protein in 0.6 ml of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). Time, temperature, amount of basement membrane, and ^{125}I-fibronectin concentration were varied as indicated to establish binding characteristics and optimum conditions for comparative studies. The tubes in which incubations were performed had been previously treated overnight with PBS containing 0.1% BSA by capping the tubes and continuously mixing them on a rotating agitator to reduce nonspecific binding to tube surfaces. Samples of basement membrane were ground to a homogeneous fine powder with mortar and pestle before being weighed individually on an analytical balance and placed in pretreated tubes that were agitated during the incubation period. The lyophilized GBM preparations were ground to maximize accessibility of potential binding sites to labeled fibronectin and to minimize differences in exposed surface areas between normal and diabetic membranes. After incubation, the basement membranes were collected by centrifugation and washed at least 10 times with the PBS solution, and the radioactivity was measured in a Mini γ-counter (Mini Instruments, Essex, UK). Control tubes contained radiolabeled protein without basement membrane to allow correction for nonspecific binding to tube surfaces. Values for this nonspecific binding were <1% of added counts at all concentrations of ^{125}I-fibronectin employed.

RESULTS

Preliminary experiments explored the extent of washing required after incubation to ensure that ^{125}I-fibronectin associated with the basement membrane pellet represented actual binding. This was determined by counting radioactivity in the supernatant in each successive wash. As seen in Fig. 1, radioactivity in the wash progressively diminished, reaching constant levels after 7–8 washes. In subsequent experiments, all pellets were therefore washed 10 times before counting. Under these conditions, incubation of GBM with ^{125}I-fibronectin resulted in binding of the radiolabeled protein that showed a direct relationship to the amount of basement membrane whether from control (Fig. 1) or diabetic animals. Confirmation that radioactivity remaining with the membrane pellets represented ^{125}I-fibronectin bound to GBM was achieved by subjecting reacted samples to SDS-PAGE followed by fluorography, which demonstrated that the radiolabel was associated with high-molecular-weight GBM protein. Furthermore, the radioactive areas were shown to contain fibronectin by transferring proteins from the gels to nitrocellulose, followed by immunoblotting with anti-fibronectin antibody (Bethesda Research, Gaithersburg, MD) and

TABLE 1
Effect of additives (50 μg each) on ^{125}I-fibronectin binding to glomerular basement membrane (GBM)

Addition	Bound cpm	Change in binding (%)
None	13,700	
Fetuin	14,200	+3.6
IgG (human)	13,360	−2.5
IgG (rat)	10,850	−20.8
α_1-Acid glycoprotein	12,265	−10.5
Chicken egg albumin	13,600	−0.7
Bovine glycoprotein	13,000	−5.1
Unlabeled fibronectin	3480	−74.6

Each incubation performed with 1 mg GBM for 3 h with 125 ng ^{125}I-fibronectin and additives in 1.2 ml PBS containing 0.1% BSA. Control incubations performed with GBM and ^{125}I-fibronectin alone. Percent change in binding calculated from bound radioactivity in experimental versus control incubations after subtraction of appropriate blanks for nonspecific adherence. Radioactivity in the GBM pellet from incubations performed without additives was set at 0% change for each assay.

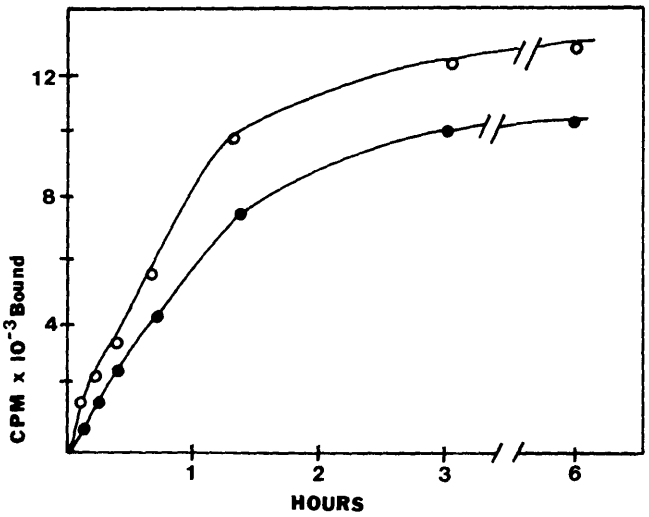

FIG. 2. Binding of ^{125}I-fibronectin to glomerular basement membrane (GBM) as function of time. In each series of experiments, 1 mg GBM was incubated with 60 ng of ^{125}I-fibronectin in 0.6 ml of PBS containing 0.1% BSA at room temperature for times indicated. Control incubations were conducted without basement membrane to correct for nonspecific adherence to tube surfaces. Binding was measured as radioactivity bound to membranes after 10 washes with rapid centrifugation. Results are expressed as cpm bound with control (●) and diabetic (○) membranes. Data are representative of 4 separate experiments.

reaction with peroxidase-labeled second antibody (goat anti-rabbit IgG) and substrate. The specificity of this binding was established by demonstrating that the binding of ^{125}I-fibronectin was effectively blocked when unlabeled fibronectin (50 μg) was added together with the radiolabeled protein, whereas the addition of similar amounts of other proteins had little or no influence on binding (Table 1). Because ^{125}I-fibronectin binding in the presence of a 400-fold excess of unlabeled fibronectin was 25% that bound in the absence of unlabeled fibronectin, specific binding represented ~70–80% of total binding.

Binding was also time dependent, progressively increasing between 5 and 100 min of incubation and changing little beyond 180 min, for up to 6 h of incubation studied (Fig. 2). The amount of binding varied with temperature, being maximum at 25°C compared to 5°, 10°, or 37°C (Fig. 3). Subsequent incubations comparing ^{125}I-fibronectin binding to control versus diabetic basement membranes were therefore conducted at 25°C for 3 h.

With 1 mg of GBM and between 60 and 1600 ng of ^{125}I-fibronectin, binding was dose dependent (Fig. 4). The amount bound continued to increase in proportion to the amount of radiolabeled protein added to the incubation mixture up to ~1600 ng, which was the maximum concentration studied. This lack of saturability suggests that once initiated, fibronectin binding to GBM promotes the attachment of additional fibronectin molecules, presumably by the formation of multimers.

Treatment with up to 4.0 M NaCl removed only small amounts of bound radioactivity, indicating that most of the association was nonionic (Table 2). On the other hand, treatment with reducing agent removed ~25% of the bound radioactivity, indicating that at least some of the binding results from disulfide linkages (Table 2). This finding coupled with the lack of saturability presented above suggested that, once bound to GBM, fibronectin can self-associate via disulfide bonding. This point was corroborated directly with SDS-PAGE followed by fluorography of reacted samples that had been subjected to denaturation alone versus reduction and denaturation in SDS before electrophoresis. In the former samples, radiographic areas were localized to the highest-molecular-weight regions, just penetrating 7% gels, especially when derived from incubations with higher (>500 ng) concentrations of ^{125}I-fibronectin; in contrast, radiographic exposure in reduced and denatured samples was prominent in gel areas corresponding to 200,000–400,000 M_r. Findings were similar when gels containing nonreduced or reduced specimens were transferred to nitrocellulose, immunoblotted with anti-fibronectin antibody, and reacted with peroxidase-labeled second antibody and substrate; in these experiments, unreduced GBM-reacted ^{125}I-fibronectin migrated in the highest-molecular-weight region, whereas fibronectin in reduced samples exhibited electrophoretic mobility in the 200,000- to 400,000-M_r region. However, it should be noted that the electrophoretic pattern of GBM components is also different under nonreducing versus reducing conditions, showing high-molecular-weight bands with denaturation alone and components of 180,000 M_r or less with reduction plus denaturation.

Pretreatment of the basement membrane with protease-free collagenase (Advance Biofactors) decreased binding by ~60%, whereas pretreatment with chondroitinase ABC plus heparinase (Sigma, St. Louis, MO) decreased binding by ~23% (Table 3). Similar results were obtained with different concentrations (50–200 μg) of collagenase or with variation of the amount of glycosaminoglycan-degrading enzymes over a twofold range. Thus, if specific binding represents 70–80% of the total, virtually all of the ^{125}I-fibronectin bound to GBM could be accounted for by binding to collagen and proteoglycan components of the matrix. However, it

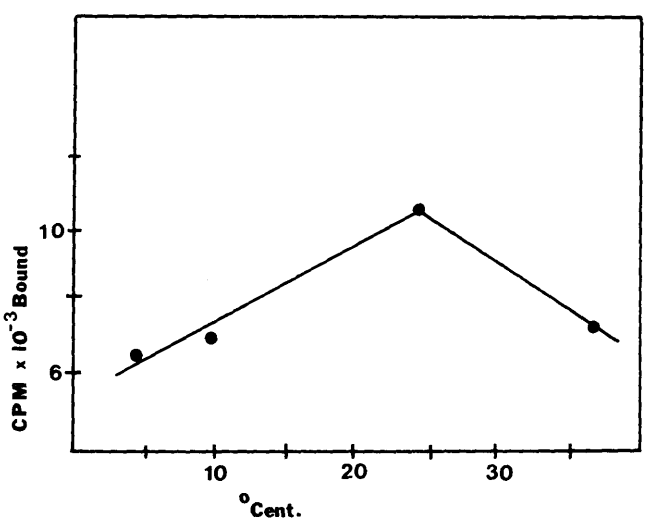

FIG. 3. Binding of ^{125}I-labeled fibronectin to glomerular basement membrane (GBM) as function of temperature. Incubation conducted with 1 mg GBM and 60 ng ^{125}I-fibronectin for times indicated. Bound radioactivity measured as described in Fig. 2 legend. Data are representative of 3 separate experiments.

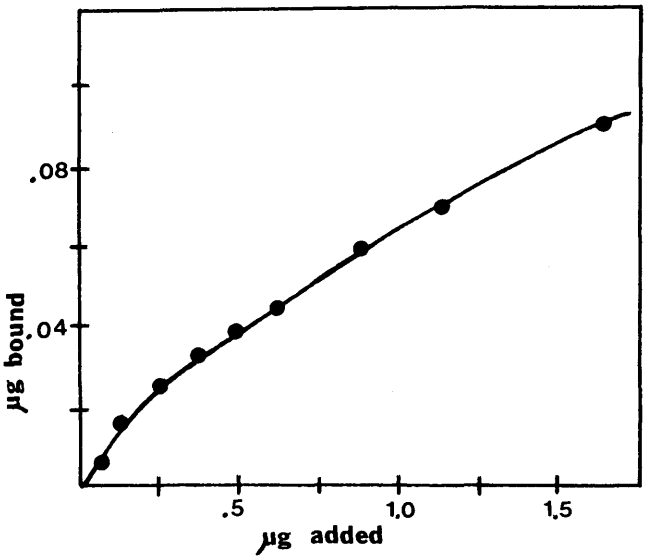

FIG. 4. Binding of ^{125}I-fibronectin as function of fibronectin concentration. In each assay, 1 mg of glomerular basement membrane was incubated for 3 h with indicated amount of radiolabeled protein. Control incubations conducted without basement membrane were performed for each concentration of added fibronectin, and results were corrected accordingly for nonspecific adherence to tube surfaces. Data are representative of 4 separate experiments.

should be noted that commercial preparations of chondroitinase or heparinase may be contaminated with small amounts of protease (<1% according to the manufacturer), and thus the relative amount of fibronectin binding to proteoglycan components may be overestimated. That binding was depressed to ~25% of control values when incubations were conducted in the presence of 1–2 mg gelatin supports the interpretation that collagenous components of GBM are largely responsible for ^{125}I-fibronectin binding.

Parallel incubations with normal or diabetic GBM revealed that preparations from diabetic animals consistently bound more radiolabeled fibronectin then did those from control rats. This was observed at all times examined (Fig. 2) and with amounts of basement membrane varying from 0.5 to 2.0 mg/incubation. After 3 h of incubation with 1 mg of GBM and 60 ng of ^{125}I-fibronectin, binding was significantly greater in diabetic than in control samples (7.99 ± 0.83 vs. 6.55 ± 1.19, mean ± SD, respectively; Fig. 5). Specific binding was also greater in diabetic than in control samples, as demonstrated by repeating these incubations in the presence of excess (75 µg) unlabeled fibronectin (6210 ± 1134 vs. 8622 ± 1150 cpm bound/mg GBM, normal vs. diabetic, respectively). ^{125}I-fibronectin bound to diabetic GBM was also partially removed by treatment with reducing agent, although the relative reduction in binding at comparable concentrations of β-mercaptoethanol was less than that observed with control basement membrane (Table 2).

DISCUSSION

The ability of fibronectin to bind to various collagen types is well established (21,30–34), and its interaction with denatured type I collagen has been exploited to develop a rapid and efficient method of isolation of fibronectin from plasma (27). Although there has been no direct evidence that fibronectin binds to basement membranes, the report that type IV collagen, which is the principal collagenous component of these extracellular matrices, inhibits fibronectin binding to type I collagen suggested such an interaction (21). In our experiments, ^{125}I-fibronectin bound to a natural basement membrane in a time-related and dose-dependent manner. Because the GBM preparations used in these experiments are devoid of cell surface components, fibronectin binding to this matrix probably represents covalent association rather than classic receptor binding. This interpretation is supported by our observation that the addition of unlabeled fibronectin (50 µg) after 3 h of incubation displaced only 26% of the bound ^{125}I-fibronectin, in contrast to the 75% inhibition of binding that occurred when labeled and unlabeled fibronectin were incubated simultaneously. This finding further suggested that part of the ^{125}I-fibronectin binding to GBM is functionally irreversible. Additionally, the lack of saturability argues against receptor-mediated binding and

TABLE 2
Effect of treatment with reducing agent or salt on fibronectin binding to glomerular basement membrane (GBM)

Sample	Bound cpm	Treatment	Remaining cpm bound	Change in binding (%)
Control	9808		9705	−1.0
	7555	20 mM MSH	5960	−21.1
	11,312	40 mM MSH	8682	−23.2
	10,507	100 mM MSH	7629	−27.4
	9857	500 mM MSH	6102	−38.1
Diabetic	13,999		13,818	−1.3
	13,693	20 mM MSH	11,015	−19.6
	13,231	40 mM MSH	11,397	−13.9
	13,794	100 mM MSH	11,023	−20.1
	13,692	500 mM MSH	10,546	−23.0
Control	7894	.5 M NaCl	7553	−4.3
	8923	1.0 M NaCl	8493	−4.8
	8493	2.0 M NaCl	7824	−7.8
	6709	4.0 M NaCl	6224	−7.2

Each incubation performed in duplicate with 1 mg GBM for 3 h with ≈95,000 cpm (60 ng) of ^{125}I-fibronectin, after which the basement membranes were collected, washed 10 times, and counted. For treatment with β-mercaptoenthanol (MSH), the membranes were then incubated for 20 min in 25 mM Tris buffer (pH 7.4) containing the indicated concentration of MSH, centrifuged and washed twice with Tris buffer, and again counted. For treatment with NaCl, the centrifuged membranes were washed twice with the indicated concentrations of NaCl and again counted.

is consistent with the described ability of plasma and cellular fibronectins to form high-molecular-weight multimers in extracellular matrices (35) as well as with our findings that the association is nonionic and is partially due to disulfide linkages. Fibronectin binds to both collagenous and glycosaminoglycan components of the GBM, although the former appears to account for ~75% of the specific binding. It is likely that specific domains in the type IV collagen, as has been shown for type I collagen (21,34), contained within the collagenous portion are responsible for this component of the binding of fibronectin to GBM.

Glomerular basement membrane purified by osmotic lysis and sequential detergent extraction, as used in these experiments, contains peripheral and mesangial basement membrane material. Whether fibronectin binding primarily occurs at peripheral versus mesangial sites cannot be discerned from our results. However, because major antigenic components (type IV collagen, laminin, proteoglycan) are shared by both loci (36), it is likely that fibronectin binds to collagen and glycosaminoglycan in both peripheral and mesangial matrix.

The increased binding of ^{125}I-fibronectin to GBM isolated from diabetic rats is of interest from several perspectives. First, the increase may reflect a qualitative or quantitative biochemical change in the GBM in diabetes (37–39). Second, it may help explain the finding with immunocytochemical techniques that fibronectin is increased in the mesangium of glomeruli in diabetic patients before the onset of renal insufficiency (36). Third, in view of the ligand-binding and cell-matrix attachment functions of fibronectin, its increased binding to diabetic GBM may influence functional and/or organizational properties of this extracellular matrix in diabetes. Fourth, the fact that diabetic GBM binds more fibronectin than normal reveals a unique difference in the two preparations that has not surfaced with numerous other types of comparative analyses. Whether this difference is due to differences in chemical composition, relative amounts of macromolecular components, or conformational features remains to be established. Finally, the apparently dichotomous effects of diabetes on different aspects of matrix-ligand interactions raise new questions concerning the relative pathophysiological importance of alterations in these interactions that may be associated with the diabetic state. Thus, the decreased binding of fibronectin to gelatin (denatured collagen) and heparin induced by excess nonenzymatic glycosylation must be reconciled with the increased fibronectin-binding property that is conferred on the assembled extracellular matrix subjected in vivo to a hyperglycemic milieu for several weeks.

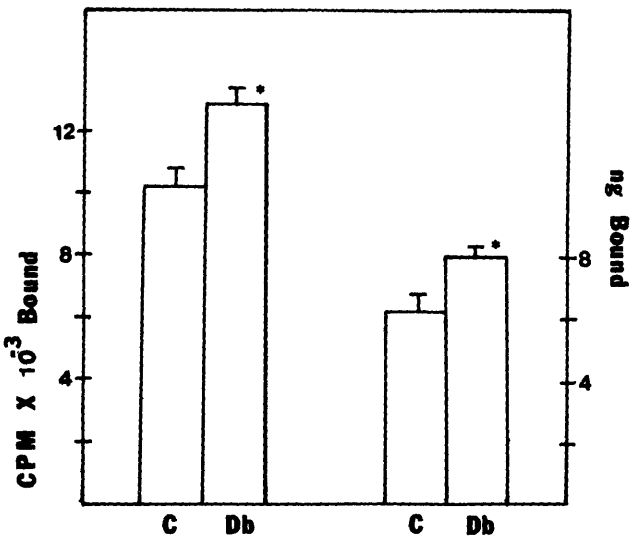

FIG. 5. ^{125}I-fibronectin binding to control and diabetic basement membranes. Incubations conducted with 1 mg glomerular basement membrane (GBM) and 60 ng of radiolabeled fibronectin for 3 h at room temperature. Results are expressed as cpm (*left*) and ng (*right*) bound and represent means ± SE of values obtained with incubations of 13 control and 12 diabetic samples derived from 6 separate GBM preparations from each group. (Standard deviations for ng bound are given in text; for cpm bound, values are 10,336 ± 1790 and 12,913 ± 1320, control and diabetic, respectively.) *$P < .05$ compared with control by Student's *t* test.

TABLE 3
Effect of enzyme treatment on fibronectin binding to glomerular basement membrane (GBM)

Treatment	Bound cpm	Change in binding (%)
None	7040 ± 55	
Collagenase	2546 ± 154	−63.8
Heparinase	6388 ± 75	−9.3
Chondroitinase	6143 ± 98	−12.7
Heparinase + chondroitinase	5396 ± 86	−23.4

Incubations performed for 3 h with ≈90,000 cpm (60 ng) of ^{125}I-fibronectin. For enzyme treatment, individual samples of 1 mg of GBM were digested overnight at 37°C with protease-free collagenase (Advance Biofactors; 100 μg in 0.025 M Tris, pH 7.4, containing 0.01 M $CaCl_2$), chondroitinase ABC (2 U in 0.01 M Tris, pH 7.4), and/or heparinase (5 U in 0.01 M Tris, pH 7.4). The basement membrane remaining undigested after such treatment was collected by centrifugation, washed, and incubated with ^{125}I-fibronectin. Results represent means ± SE of 4 experiments.

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