Selective Deglycosylation of the Heparan Sulfate Proteoglycan of Bovine Glomerular Basement Membrane and Identification of the Core Protein*

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The heparan sulfate proteoglycan of the bovine glomerular basement membrane ($M_r = 200,000$, 30% carbohydrate by weight) has been deglycosylated by various chemical and enzymatic procedures to identify the core protein and provide information about the N- and O-linked saccharide units. Heparitinase digestion of the proteoglycan reduced its $M_r$ to 143,000, consistent with the removal of its four glycosaminoglycan chains with the exception of short segments adjacent to the carbohydrate-protein linkage region, whereas nitrous acid treatment brought about a smaller reduction in size ($M_r = 188,000$) which was shown to be due to the resistance of the internal portion of the heparan sulfate polymer to this reagent. Incubation of the heparitinase-digested proteoglycan with peptide N-glycosidase F decreased its $M_r$ by about 8,000 and liberated oligosaccharides which were primarily acidic in nature; since endo-$eta$-$N$-acetylgalactosaminidase H did not bring about any saccharide release, it appears that the N-linked carbohydrate units (three per molecule) occur exclusively as the complex type.

Treatment of the proteoglycan with trifluoromethanesulfonic acid, a reagent which cleaves all saccharide units, yielded the core protein which migrated as a single discrete band ($M_r = 128,000$) on polyacrylamide gel electrophoresis. Although the native and heparitinase-treated proteoglycan reacted with concanavalin A and Bandeiraea simplicifolia I, the core protein had no affinity for these lectins, and this loss of reactivity can be attributed to the removal of the N- and small O-linked saccharides. However, the immunoreactivity of the deglycosylated protein with antisera directed against the intact proteoglycan was to a large measure preserved, suggesting that the polyclonal response to this glomerular basement membrane glycoconjugate is primarily directed against determinants on the polypeptide portion.

The GBM,¹ which functions as the primary constituent of the renal filtration apparatus (1, 2), is an integrated multicomponent structure consisting of collagen, proteoglycan, and several glycoproteins. Its permeability is believed to be defined by pore size and electrostatic charge, and the latter property has been suggested to be due primarily to anionic groups present on protein-linked heparan sulfate chains (3).

Previous studies carried out in our laboratory have indicated that most of the hexuronic acid of bovine GBM occurs in a heparan sulfate proteoglycan with a molecular weight of 200,000 which contains four glycosaminoglycan chains ($M_r = 14,000$) as well as several N-linked and small O-linked carbohydrate units (4). However, the major part of this glycoconjugate (70% by weight) was found to consist of polypeptide (4); this portion of the molecule is of considerable interest since it is likely that in addition to providing sites for the attachment of the various saccharide chains it may play an important role in the interaction of the proteoglycan with other basement membrane macromolecules and contribute substantially to its antigenicity.

In the present investigation, by applying enzymatic and chemical deglycosylation procedures to the bovine GBM proteoglycan, it has been possible to selectively remove or shorten various carbohydrate chains, and this has led to the identification of the core protein. Moreover, additional information about the saccharide units was obtained from a study of the deglycosylation products as well as from an assessment of the interaction of native and modified proteoglycan with concanavalin A and Bandeiraea simplicifolia I lectins. An evaluation of the immunological reactivity of the deglycosylated proteoglycan indicated that polyclonal antibodies raised against the native molecule are primarily directed toward its polypeptide portion.

EXPERIMENTAL PROCEDURES

Preparation of GBM Heparan Sulfate Proteoglycan—Basement membranes obtained from bovine renal glomeruli were extracted with 4 M guanidine HCl as previously described (4), and the solubilized proteoglycan was purified by DEAE-cellulose chromatography (4) employing a modified sodium chloride elution gradient (2).

Preparation of Heparan Sulfate Chains—After treatment of the proteoglycan (2 mg) with 0.2 ml of 0.1 N NaOH containing 1 M NaBH₄ (Du Pont-New England Nuclear, adjusted to 110 mCi/mmol) for 72 h at 37 °C, the reduced radiolabeled glycosaminoglycan chains were purified by Bio-Gel filtration and DEAE-cellulose chromatography as previously reported (4).

Radioiodination of Proteoglycan—Reductive alklylation of the proteoglycan was performed with $[^3]C$-formaldehyde (Du Pont-New England Nuclear, 52 mCi/mmol) as described (4) and resulted in a product with a specific activity of 8.1 x $10^6$ dpm/mg.

TMS Treatment of Proteoglycan—Deglycosylation of the $[^3]C$-labeled proteoglycan (1.2 x $10^6$ dpm) as well as this unlabeled glycoconjugate was performed with 18 U.S.C. Section 1754 solely to indicate this fact.

1 This work was supported by Grant AM 17325 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

2 Recipient of a Capps Scholarship in Diabetes from Harvard Medical School.

* This lectin is also known as Griffonia simplicifolia I.
conjugate (0.5 mg) was accomplished in 100 μl of TFMS-anisole (2:1, v/v) reagent (5) under nitrogen for 3 h at 0 °C. Neutralization and ether extraction were carried out as previously reported (5), followed by dialysis against 20 mM ammonium bicarbonate; the yield after these procedures was 60% of the starting material.

**Nitric Acid Treatment of Proteoglycan and Free Heparan Sulfate Chains**

After labeled (0.5 mg) and unlabeled (1.3 × 10^10 dpm) proteoglycan as well as the NaBH₄-reduced glycosaminoglycan chains (4 × 10^10 dpm) were treated with nitric acid reagent as previously described (6). After this procedure the proteoglycan samples were dried and extracted three times with 80% ethanol, whereas those containing the free heparan sulfate chains were applied to a column (1.8 × 197 cm) of Bio-Gel P-10 (100–200 mesh) equilibrated with 0.1 M pyridine acetate, pH 5.0, at a flow rate of 12 ml/h.

**Heparitinase and Heparanase Digestions**—Radiolabeled and unlabeled proteoglycan (1.8 × 10^10 dpm or 0.5 mg, respectively) as well as NaBH₄-reduced heparan sulfate chains (4 × 10^10 dpm) were incubated in 100 μl of 0.05 M Tris- HCl buffer, pH 7.2, containing 0.02 M calcium acetate for 16 h at 37 °C with either 10% milliliters of heparitinase or heparanase from *Flavobacterium heparinum* (Miles Laboratories) in the presence of toluidine; in the case of the proteoglycan, 1 mM phenylmethylsulfonl fluoride and 10 μg each of antipain, leupeptin, phosphoramidone, elastatinase, pepstatin, amastatin, and bestatin were included in the incubation to serve as protease inhibitors (7). The digestions were terminated by heating at 100 °C for 2 min; proteoglycan was desalted on a column of Bio-Gel P-2 (1 × 25 cm) equilibrated in 0.05 M ammonium bicarbonate, whereas samples containing the heparan sulfate chains were fractionated on Bio-Gel P-10.

**Peptide N-Glycosidase F and Endo H Digestions**—Radiolabeled proteoglycan (2 × 10^10 dpm), both before and after heparitinase digestion, was digested with 0.26 units peptide N-glycosidase F from *Flavobacterium meningosepticum* (Genzyme, N-glycanase) in 30 μl of 0.1 M sodium phosphate, pH 8.6, containing 0.17% SDS, 0.33% 2-mercaptoethanol, 6 mM EDTA, 0.6% Nonidet P-40, and 2 mM phenylmethylsulfonl fluoride or 10 μl milliliters of endo H (*Streptomyces griseus*, Miles Laboratories) in 100 μl of 0.15 M sodium citrate, pH 5.2, containing 0.1% bovine serum albumin and either 0.05% SDS or 0.5 M sodium thiochyanate (8). Incubations were carried out at 57 °C for 6 h in the presence of toluidine; subsequently, the samples were treated at 100 °C for 2 min, and aliquots were removed for examination by polyacrylamide gel electrophoresis.

In order to characterize the oligosaccharides released by enzyme action, unlabeled heparitinase-digested proteoglycan (50 μg) was incubated with the endoglycosidases under the conditions already specified. After removal of salt by passage of the peptide N-glycosidase F digest through a column of Bio-Gel P-2 (equilibrated in 0.1 M pyridine acetate, pH 5.0) and of the endo H-treated sample through coupled columns of Dowex 50-X2 (200–400 mesh, H⁺ form) and Dowex 1-X2 (200–400 mesh, acetate form), the liberated oligosaccharides were reduced with NaBH₄ (1 mCi, 7.5 Ci/mmol) in 100 μl of 0.2 M sodium borate buffer, pH 9.0, for 4 h at room temperature. The reaction was terminated by addition of acetic acid as acetic acid/water/acetate acid (5:5:5,1) of the molecule's weight was reduced with NaBH₄ (1 mCi, 7.5 Ci/mmol) in 100 μl of 0.2 M sodium borate buffer, pH 9.0, for 4 h at room temperature. The reaction was terminated by addition of acetic acid as acetic acid/water/acetate acid (5:5:5,1) of the molecule's weight.

**RESULTS**

**Identification of the Bovine GBM Heparan Sulfate Proteoglycan Core Protein**—Treatment of the ¹⁴C-labeled proteoglycan with TFMS resulted in a reduction of its apparent molecular weight from 200,000 to 128,000 as determined by SDS-polyacrylamide gel electrophoresis (Fig. 1). This decrease in mass was consistent with the removal of the various carbohydrate units which together have been reported to account for 30% of the molecule's weight (4). Moreover, the pronounced sharpening of the electrophoretic band brought about by the TFMS treatment (Fig. 1) indicated that the broad

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**FIG. 1. Comparison of the effects of various saccharide removal procedures on the migration of GBM heparan sulfate proteoglycan during polyacrylamide gel electrophoresis in SDS.** The ¹⁴C-labeled proteoglycan (9,000 dpm) was electrophoresed directly (Native) or after treatment with nitrous acid (HNO₂), heparitinase (HSase), or TFMS as described under "Experimental Procedures." The components were visualized by fluorography; the designated molecular weight markers were mouse laminin α-subunit (200,000), *Escherichia coli β-galactosidase* (116,000), bovine serum albumin (66,000), hen ovalbumin (45,000), and bovine erythrocyte carbonic anhydrase (29,000).

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appearance of the native proteoglycan on the polyacrylamide gel is due to variability in its saccharide chains.

Comparison of the Effect of Nitrous Acid and Heparitinase Treatment on the Proteoglycan and Its Glicosaminoglycan Chains—Nitrous acid degradation of the native proteoglycan reduced its apparent molecular weight to approximately 168,000, whereas heparitinase digestion brought about a more substantial change with the appearance of a component with an $M_r$ of 143,000 (Fig. 1). The product of this enzymatic treatment migrated on electrophoresis to a position intermediate between that of the nitrous acid-degraded and the TFMS-deglycosylated proteoglycan, indicating that the four (4) heparan sulfate chains (4) had been substantially shortened. However, in contrast to the pronounced effect brought about by heparitinase, digestion of the proteoglycan with heparinase did not result in a detectable change in its electrophoretic migration (data not shown).

In order to correlate the molecular weight changes brought about by nitrous acid and heparitinase on the intact proteoglycan to cleavages occurring within the heparan sulfate chains, the effect of both of these treatments on the isolated NaB$_3$H$_4$-reduced glycosaminoglycans was evaluated. As anticipated from a previous study (4), these chains, which terminate in radiolabeled [3H]xylitol, were converted by nitrous acid treatment from material which eluted ($K_{av} = 0.07$) near the void volume of a Bio-Gel P-10 column to fragments which were included in such a column; most of the radioactivity (73%) emerged as a major peak ($K_{av} = 0.35$), although a spectrum of smaller components was also evident (Fig. 2). Digestion of the glycosaminoglycan chains with heparitinase effected a more pronounced reduction in molecular weight, and a peak which represented the short stubs from the reducing end eluted with a $K_{av}$ of 0.64 (Fig. 2). The heparan sulfate chains, like the intact proteoglycan, were found to be resistant to the action of heparinase (data not shown).

Release of N-Linked Carbohydrate Units from Proteoglycan by Peptide N-glycosidase F Treatment—Digestion of the heparitinase-treated proteoglycan with peptide N-glycosidase F, an enzyme which cleaves both mannose and an complex N-linked saccharide units (16), resulted in a further reduction in its molecular weight ($M_r = 135,000$). The product of this treatment, although migrating faster than the proteoglycan digested only with heparitinase, still moved more slowly than the TFMS-deglycosylated molecule (Fig. 3). Incubation of the native proteoglycan with peptide N-glycosidase F brought about no detectable change in molecular weight (data not shown).

In contrast to peptide N-glycosidase F, endo H digestion of the heparitinase-treated proteoglycan failed to bring about any change in electrophoretic migration when either sodium thiocyanate or SDS were employed as denaturants. Furthermore, no released polyanomannose oligosaccharides (Man$_n$GlcNAc to Man$_4$GlcNAc) were evident upon thin layer chromatographic examination of NaB$_3$H$_4$-reduced products under conditions in which less than 0.03 nmol of oligosaccharide/nmol of protein were clearly evident in concurrent digestions of calf thyroglobulin (17); even endo H treatment of pronase-digested proteoglycan (18), to eliminate potential steric interference, failed to result in a detectable release of carbohydrate units.

The oligosaccharides released by peptide N-glycosidase F could be resolved after NaB$_3$H$_4$ reduction by Bio-Gel P-4 filtration into three peaks with average molecular weights of 4700, 3100, and 2300, respectively, which were present in molar ratios of 0.5:1.00.4 (data not shown). When the components in the peak of intermediate size ($M_r = 3100$) were further fractionated on DEAE-cellulose, charge heterogeneity comparable to that observed in fetuin N-linked saccharide units (19) was evident (Fig. 4). Thin layer chromatography after re-N-acetylation of the hexosamines released by acid hydrolysis of the peptide N-glycosidase F-liberated oligosaccharides indicated, as anticipated, that radiolabeled N-acetylglucosaminitol was in the terminal position (data not shown).

Effect of Deglycosylation on the Interaction of Proteoglycan with Lectins—With the use of a sensitive solid-phase assay which employs anti-lectin sera and radiolabeled protein A to

![Fig. 2. Comparison of the effects of nitrous acid and heparitinase treatment on the heparan sulfate chains of the GBM proteoglycan as determined by filtration on Bio-Gel P-10. The NaB$_3$H$_4$-reduced glycosaminoglycan (4 x 10$^7$ dpm) was applied to the column (1.8 x 127 cm) before (- - -) and after treatment with nitrous acid (O) or heparitinase (•). The column was eluted with 0.1 M pyridine acetate, pH 5.0, at a flow rate of 12 ml/h. $V_0$ and $V_f$ refer, respectively, to the void and total volumes of the column; $K_S$ designates the elution position of keratan sulfate glycopeptide (average $M_r = 10,000$).](image)

![Fig. 3. Effect of peptide N-glycosidase F treatment on the migration of GBM heparan sulfate proteoglycan during polyacrylamide gel electrophoresis in SDS compared to other deglycosylation procedures. The 14C-labeled proteoglycan (9000 dpm) was applied to the gel after heparitinase (HSase), TFMS, or heparitinase plus peptide N-glycosidase F (HSase + N-Glyase) treatment as described under “Experimental Procedures.” The components were visualized by fluorography; the molecular weight markers were the same as in Fig. 1.](image)
Deglycosylation of Glomerular Basement Membrane Proteoglycan

Fig. 4. Chromatography on DEAE-cellulose of NaBH₄-reduced oligosaccharides released from GBM proteoglycan by peptide N-glycosidase F. The major saccharide peak (average Mₐ = 3,100) obtained by filtration on Bio-Gel P-4 was applied (10,000 dpm) to the column equilibrated with 5 mM pyridine formate, pH 5.0, and after a wash with this buffer elution was carried out with a linear gradient as described under “Experimental Procedures.” The arrows indicate the elution of N-acetylated hydrazine-released N-linked carbohydrate units from fetuin containing, respectively, 1, 2, 3, and 4 sialic acid residues.

Fig. 5. Evaluation of the reactivity of the GBM proteoglycan with lectins as determined by solid-phase immunoassay. The binding of concanavalin A (Con A) and B. simplicifolia I (BS-I) to micro-wells coated with increasing amounts of proteoglycan (PG) as well as standard glycoproteins, ovalbumin (OV), laminin (LM), fetuin (Fet), and thyroid GP-1 (GP-1) was determined by an assay described under “Experimental Procedures” in which anti-lectin serum followed by ¹²⁵I-labeled protein A are employed. To test the specificity of the lectin interaction, the proteoglycan was incubated with Con A and BS-I in the presence of 100 mM methyl-α-D-mannoside (PG + CH₃-Man) and 100 mM methyl-α-D-galactoside (PG + CH₃-Gal), respectively.

Fig. 6. Effect of saccharide removal on the reactivity of the GBM proteoglycan with concanavalin A (Con A) and B. simplicifolia I (BS-I). The intact (NATIVE), heparitinase-digested (HSase), and TFMS-treated GBM proteoglycan were assayed in increasing amounts by solid-phase radioimmunoassay using ¹²⁵I-labeled protein A as described under “Experimental Procedures.”

Fig. 7. Comparison of the reactivity of native and deglycosylated proteoglycan with anti-bovine GBM heparan sulfate proteoglycan serum. Native, heparitinase-digested (HSase), and TFMS-treated GBM proteoglycan were assayed in increasing amounts by solid-phase radioimmunoassay using ¹²⁵I-labeled protein A as described under “Experimental Procedures;” the serum was employed at 1:1000 dilution.

measure lectin-glycoconjugate interaction, the binding of concanavalin A and B. simplicifolia I to the GBM proteoglycan could be demonstrated (Fig. 5). The specificity of the assay was established by noting that the appropriate methylglycoside inhibited lectin binding to proteoglycan and that a number of standard glycoproteins, namely ovalbumin (20), fetuin (21), laminin (22), and thyroid GP-1 (23), reacted with the concanavalin A and B. simplicifolia I as would be anticipated from the nature of their carbohydrate units (Fig. 5).

The lectin-binding assay provided a sensitive tool for assessing the removal of saccharides by deglycosylation procedures; the binding of both concanavalin A and B. simplicifolia I to the proteoglycan was decreased by more than 95% after TFMS treatment; in contrast, enzymatic removal of heparan sulfate chains did not significantly alter the extent of these interactions (Fig. 6).

Immunochromic Properties of Proteoglycan after Deglycosylation—As indicated by solid-phase radioimmunoassay, deglycosylation had little effect on the interaction of the proteoglycan with an antiserum raised against the native molecule (Fig. 7). The heparitinase-digested proteoglycan bound antibody to the same extent as the intact glycoconjugate, whereas...
under the conditions employed

sulfate chains, whereas heparitinase releases all but a small internal partial removal of a particular type of carbohydrate unit and a minus sign to indicate lack of effect on this unit.

The apparent molecular weights were calculated from electrophoretic migration of native and modified proteoglycan relative to standard proteins and must be considered as approximations.

Nitrous acid removes only the peripheral portion of the heparan sulfate chains, whereas heparitinase releases all but a small internal segment.

The Asn-linked GlcNAc is conserved by this procedure, and only a partial removal of Ser(Thr)-linked GalNAc residues is achieved under the conditions employed (5).

TFMS-prepared core protein retained 80% of the original activity. Immunoblotting with this anti-proteoglycan serum delineated bands in the deglycosylated preparations and in the intact proteoglycan (Fig. 8) which migrated to the same positions as those revealed by chemical radiolabeling of the peptide portion (Fig. 1).

**DISCUSSION**

By employing enzymatic and chemical deglycosylation procedures of different specificities, we have been able in the present investigation to identify the core protein of the bovine GBM heparan sulfate proteoglycan and to provide further information in regard to its various saccharide units (Table I).

Treatment of the proteoglycan with TFMS, a reagent which is known to degrade all carbohydrate units (5), yielded a single discrete polypeptide band with an apparent $M_r$ of 128,000 which can be considered to represent the core protein. Indeed, the size of this product corresponds well with that expected from compositional analyses which have shown that saccharides constitute 30% of the proteoglycan's weight. Furthermore, the loss of lectin-reactivity of the TFMS-treated molecule was consistent with effective deglycosylation.

After heparitinase digestion the $M_r$ of the proteoglycan was decreased to an extent consistent with the removal of the four ($M_r \sim 14,000$) heparan sulfate chains which have been found in each molecule (4). A smaller reduction in size was apparent after nitrous acid treatment (Table I), and this difference can be explained by the fact that the enzyme cleaves the glycosaminoglycan chains closer to the carbohydrate-protein linkage region than the chemical reagent. Indeed, the resistance of the internal segment of the heparan sulfate chains to nitrous acid degradation has been previously observed and attributed to an uneven distribution of N-sulfate groups (4, 24).

The decrease in $M_r$ of about 8900 brought about by the peptide N-glycosidase F digestion is consistent with the removal of N-linked carbohydrate units from the proteoglycan (16). These oligosaccharides are believed to be exclusively of the complex type since no release was achieved by endo H treatment, and, indeed, DEAE-cellulose chromatography indicated that they were primarily acidic species. Since there are 8–9 mol of mannose/mol of proteoglycan (4), the presence of three complex asparagine-linked saccharide units/molecule is indicated by these data. The further decline in molecular weight brought about by TFMS treatment (about 7000) most likely represents a release of O-linked saccharides, including the remaining stubs from the heparan sulfate chains (Table I); the occurrence of a number of small GalNAc-Ser(Thr)-linked carbohydrate units on this GBM proteoglycan has previously been noted (4).

It is apparent from the present study that since the GBM proteoglycan, like those from other sources (25, 26), contains saccharide units other than the glycosaminoglycan chains, the removal of only the latter does not necessarily lead to the formation of a product that represents the polypeptide core. Indeed, a difference of 16,000 in molecular weight was observed between the heparitinase and TFMS-treated proteoglycan (Table I), and the loss of lectin interaction occurred only after the chemical deglycosylation. Presumably, the concanavalin A combines with the N-linked units of the proteoglycan, whereas α-D-galactosyl residues on N- and/or O-attached saccharides are the binding sites for the B. simplicifolia I (27). The presence of the B. simplicifolia I-reactive saccharides on the GBM proteoglycan is of particular interest since it represents yet another GBM constituent besides laminin (22) and Type IV collagen (2) which has been found to contain α-D-galactosyl groups and would therefore contribute to binding capacity of basement membranes for this lectin, which was originally reported by Peters and Goldstein (28).

The preparation of heparan sulfate-depleted and TFMS-deglycosylated proteoglycan provided us with an opportunity to assess the contribution of the saccharide units to the antigenicity of the molecule. Our data indicate that the polyclonal response to the native proteoglycan is primarily directed against determinants on the polypeptide portion of the glycoconjugate, a finding that is in agreement with observa-
tions on heparan sulfate proteoglycans from the Engelbreth-Holm-Swarm sarcoma (29).

The molecular weight of the core protein of the bovine GBM heparan sulfate proteoglycan clearly differs from that of the component which has been isolated from rat glomeruli (30), since after digestion of the latter with heparitinase a reduction in \( M_r \) from 130,000 to 18,000 has been reported. The basement membrane-producing mouse Engelbreth-Holm-Swarm sarcoma has yielded a low density \( M_r > 500,000 \) and a high density \( M_r = 350,000 \) proteoglycan which upon enzymatic removal of heparan sulfate chains have been shown to yield molecules with \( M_r \) values of 350,000–400,000 and 95,000–130,000, respectively (7); in another study a molecular weight of about 10,000 has been reported for TFMS-prepared protein core of the high density glycoconjugate from the mouse tumor (29).

Since the synthesis of glycosaminoglycans is a post-translational event (31), the difference in heparan sulfate chain lengths noted in proteoglycans from various sources (4, 7, 30, 32–34) is not surprising and can be attributed to variation in enzymatic machinery and substrate availability. Although the diversity in core protein size observed in basement membrane proteoglycans might suggest a direct genetic basis, this may not necessarily be the case; as indicated by studies with Engelbreth-Holm-Swarm tumor cells, proteolytic processing of the heparan sulfate proteoglycan can occur (35), and such a mechanism may be responsible for the formation of different core proteins from a common precursor, depending on species or cellular origin. Although the core proteins identified by deglycosylation procedures of mature proteoglycans are probably not the primary gene products, they do represent the physiologically relevant polypeptides which dictate the disposition of the carbohydrate units of the proteoglycans and influence their interaction with other basement membrane macromolecules.

REFERENCES