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Isolation and Characterization of Membrane-associated Proteoglycans from Normal and Malignant Human Mammary Epithelial Cells

D CHANNE GOWDA[1], V P BHAVANANDAN and EUGENE A DAVIDSON*

Department of Biological Chemistry, The Milton S Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033, U.S.A.
[1]*On leave of absence from the Department of Chemistry, University of Mysore, Mysore, India*

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The plasma membrane-associated proteoglycans of a malignant human breast cell line (MDA-MB-231) were compared with the corresponding proteoglycans from a normal cell line (HBL-100). The labeled proteoglycans were isolated from the plasma membranes of cells grown in the presence of [^{3}H]glucosamine and [^{35}S]Na_2SO_4 by extraction with guanidine hydrochloride and subsequently purified by DEAE-ion exchange chromatography. Their structural properties were established by treatment with nitrous acid, heparitinase and chondroitinase ABC, and by gel filtration before and after alkaline β-elimination. About 18% of the proteoglycans synthesized by these cell lines were associated with the plasma membranes. The HBL plasma membranes contained 80% heparan sulfate and 20% chondroitin sulfate proteoglycans whereas MDA plasma membranes had 50% heparan sulfate and 50% chondroitin sulfate proteoglycans. The MDA plasma membrane contained two heparan sulfate proteoglycans, both having nearly the same molecular size as the two species secreted into the medium by these cells. The HBL plasma membrane also contained two hydrodynamic size heparan sulfate proteoglycans. The larger hydrodynamic size species has a slightly lower molecular size than that secreted into the medium, and the smaller hydrodynamic size species was not detectable in the medium. Even though the major chondroitin sulfate proteoglycans from MDA plasma membranes were smaller in size than those from HBL plasma membrane, a larger proportion of the glycosaminoglycan chains of the former were bigger than those from the latter.

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulfonate; ΔDi-OS, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-ene-pyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-ene-pyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluco-4-ene-pyranosyluronic acid)-6-*O*-sulfo-D-galactose; Gdn-HCl, guanidine hydrochloride; WGA, wheat germ agglutinin.

*Author for correspondence

Cell-surface proteoglycans, especially proteoheparan sulfates, have been implicated in a number of biological functions [1], including regulation of cell growth [2-5], cellular recognition [6], cell-cell adhesion [7-9], cell-matrix and cell-substratum adhesion [10], tissue differentiation [11] and transmembrane cation flux [12]. Further, qualitative and quantitative chemical changes in the polysaccharide chains have been noted after cell transformation [1]. For instance, normal cells, compared to their malignant counterparts, contain higher proportions of heparan sulfate, suggesting a possible functional role for this polysaccharide in regulation of cell growth. Cell surface chondroitin sulfate generally increases upon cell transformation [6, 13-15]. In several malignant cells and tissues, heparan sulfate with a reduced sulfate content [16-20] and lower molecular weight [20-22] has been identified. It has been suggested that such abnormalities in cell surface proteoglycans impair normal cellular function [19].

Although the proteoglycans from a wide variety of normal and malignant cells and tissues have been studied extensively [22-31], studies of the proteoglycans produced by comparable normal and malignant cells are lacking. In a previous study from this laboratory, the nature of glycosaminoglycans synthesized by one normal and two malignant human breast cell lines was described [32]. The present studies were undertaken to investigate in detail the physical and chemical characteristics of proteoglycans synthesized by normal and malignant human breast cell lines. In this report, an account of the structural characterization of the plasma membrane-associated proteoglycans of a normal (HBL-100) and a malignant (MDA-MB-231) human breast cell line is presented.

Materials and Methods

Cell Culture

HBL-100 and MDA-MB-231 cells at passage 23 and 24, respectively, were obtained from the American Type Culture Collection and were supplied in McCoy's 5a medium. After subculturing twice in this medium, they were adapted to L-15 medium (Flow Laboratories, Rockville MD, USA). Both HBL-100 and MDA-MB-231 cells were routinely grown in 16 oz plastic bottles in L-15 medium containing penicillin-streptomycin and 10% heat-inactivated fetal calf serum (Flow Laboratories). The cells were incubated at 37°C in a humid atmosphere.

Metabolic labeling of glycoconjugates was accomplished by adding the isotopically labeled precursors [^{3}H]-glucosamine hydrochloride (Amersham, Arlington Heights, IL, USA; 20 Ci/mmol; 5 μCi/ml) and [^{35}S]Na_2SO_4 (New England Nuclear, Boston, MA, USA; 12.5 μCi/ml) to logarithmically growing cells in 25 ml of a sulfate-free and reduced galactose medium. The cultures were incubated for an additional 48 h to reach confluence.

Preparation of Plasma Membranes

The overall procedure is summarized in Fig. 1. The medium from metabolically labeled cell cultures was removed, the cell layers washed twice with 50 mM sodium phosphate buffer, pH 7.2, and then treated with 0.02% EDTA in the same buffer (2 ml/bottle) for 10 min. The detached cells (2×10^7) were collected by centrifugation, and suspended in 40 ml of homogenization buffer (10 mM sodium phosphate, pH 7.4, containing 1 mM mag-

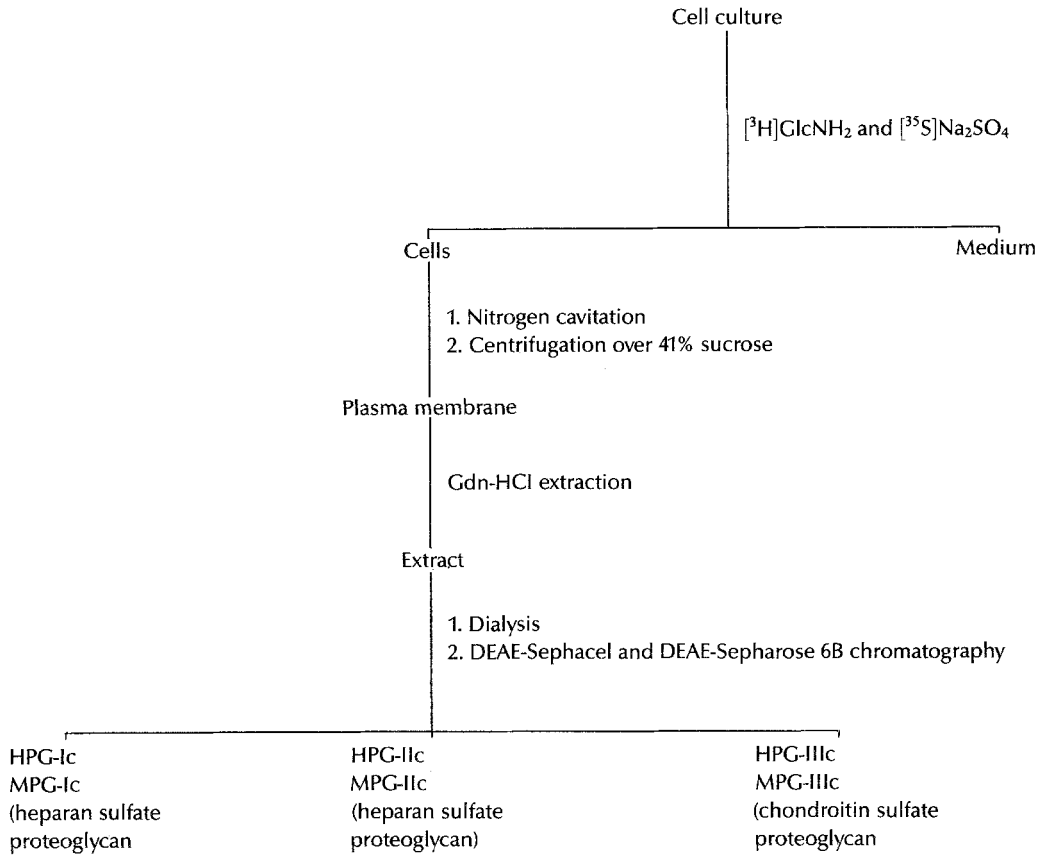

Figure 1. Isolation of plasma membrane-associated proteoglycans from HBL-100 and MDA-MB-231 cells.

nesium chloride, 30 mM sodium chloride, 1 mM dithiothreitol, 0.005 mM phenylmethylsulfonyl fluoride, 0.02% sodium azide, and 50 to 100 μg of DNase). The cells were ruptured by release from a pressurized (500 psi for 20 min) nitrogen cavitation bomb. Complete disruption of the cells was confirmed by examination of an aliquot of the homogenate by phase contrast microscopy.

The homogenate was layered on to 10 ml of a 41% sucrose solution in an UltraClear centrifuge tube which had been soaked overnight in 1 mM EDTA, pH 7.0, and rinsed thoroughly with distilled water [34]. After centrifugation at 95 000 $\times$ *g* for 1 h at 4°C in a Beckman SW27 swinging bucket rotor, the material at the interface was collected, diluted with the homogenization buffer, and centrifuged at 95 000 $\times$ *g* for 20 min at 4°C. The plasma membrane pellet was resuspended in the homogenization buffer and recentrifuged. The resulting pellet was stored at -70°C until used.

In a control experiment, plasma membranes were assayed for the following enzymes: 5'-nucleotidase [35], (Na^+, K^+) ATPase [36], glucose-6-phosphatase [37], *N*-acetylglucosaminidase [38] and succinate dehydrogenase [39]. The first two enzymes are markers for the plasma membrane, the others are specific for endoplasmic reticulum, lysosomes, and mitochondria respectively. Protein was determined by the Lowry method [40].

Extraction of Proteoglycans from Plasma Membranes

Plasma membranes from three preparations were pooled and extracted at 4°C with 5 × 2 ml portions of 50 mM Tris-HCl, pH 7.4, containing 4 M Gdn-HCl, 1 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, 5 mM benzamidine, 10 mM EDTA, 20 mM 6-aminohexanoic acid, and 1% (w/v) Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA).

Dissociative Cesium Chloride Density Gradient Centrifugation

Proteoglycan samples were dissolved in 50 mM sodium acetate, pH 6.0, containing 4 M Gdn-HCl, and solid cesium chloride was added to a concentration of 1.42 g/ml. Ultracentrifugation was performed in a Beckman SW50.1 swinging bucket rotor at 37 000 rpm (130 000 × *g*) for 48 h at 10°C as described by Yanagishita and Hascall [39]. Ten to twelve fractions of equal volume were collected from the bottom of the tube and analyzed for radioactivity and cesium chloride density.

Fractionation of Proteoglycans by DEAE-Sephacel Chromatography

The membrane extract was dialyzed at 4°C against 50 mM sodium acetate, pH 7.0, containing 6 M urea, 1 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide and 5 mM benzamidine. The solution was applied to a DEAE-Sephacel column (0.8 × 8 cm) equilibrated with 50 mM sodium acetate, pH 6.0, containing 0.5% CHAPS [23]. The column was washed with five bed volumes of the same buffer containing 0.15 M sodium chloride and then eluted with a linear gradient of 0.15 M to 1.2 M sodium chloride in the same buffer (total volume 140 ml). Fractions of 2 ml were collected and aliquots were analyzed for radioactivity.

Purification of Proteoglycans by Chromatography on DEAE-Sepharose 6B

Proteoglycan fractions obtained by DEAE-Sephacel chromatography were dialyzed against 50 mM Tris-HCl, pH 7.6, containing 6 M urea, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, 5 mM benzamidine and 0.5% CHAPS. The nondialyzable material was applied on to a DEAE-Sepharose 6B column (0.8 × 10 cm) equilibrated with 50 mM Tris-HCl, pH 7.6, containing 6 M urea and 0.5% CHAPS, and eluted with a linear gradient of 0-1.5 M lithium chloride in the same buffer (140 ml). Fractions of 2 ml were collected and aliquots analyzed for radioactivity.

Enzymatic Digestions

Proteoglycan samples were mixed with appropriate carriers (100-200 μg of heparin or chondroitin sulfate, Sigma) and treated with one or more enzymes under conditions sufficient for the complete degradation of the respective carriers. Chondroitinase ABC and chondroitinase ACII digestions were performed according to the method of Saito *et al.* [40]. Heparitinase digestion was carried out for 24 h at 43°C in 300 μl of 0.1 M sodium acetate, pH 7.0, containing 1 mM calcium acetate [42]. Chondroitinase ABC-derived disaccharides were treated with chondro-4-sulfatase or chondro-6-sulfatase for 24 h at 37°C in 60 mM sodium acetate, pH 7.5 [41]. Sialidase treatment was carried out for 24 h at 38°C in 50 mM sodium acetate buffer, pH 5.6, containing 2 mM calcium chloride [42].

WGA-Sepharose Affinity Chromatography

Wheat germ lectin was isolated from raw wheat germ according to the procedure of Nagata *et al.* [50]. Coupling of the lectin to Sepharose 4B gel was carried out by cyanogen bromide activation as described by March *et al.* [51]. The proteoglycan digest was applied on to a WGA-Sepharose (5 mg lectin/ml gel) column (0.8 × 10 cm). After washing the column with 50 mM Tris-HCl, pH 8.0, the bound material was eluted with 0.1 M *N*-acetylglucosamine in the same buffer. One ml fractions were collected and analyzed for radioactivity. The activity of the WGA-Sepharose was tested by chromatographing a modified [^{3}H-NeuAc]-α_1-acid glycoprotein prepared in this laboratory by the procedure of Van Lenten and Ashwell [52]. This product bound to the gel and was quantitatively recovered in the sugar eluate.

Paper Chromatography

The disaccharides obtained by Bio-Gel P-4 gel filtration of chondroitinase ABC-digested proteoglycans were examined by paper chromatography before and after chondrosulfatase digestion using *n*-butanol/acetic acid/1 M NH_4OH, 2/3/1 by vol, as solvent [43]. To identify the radiolabeled components on the chromatogram, the paper was cut into 1 cm strips, extracted with 1 ml water and the radioactivity was measured.

Nitrous Acid Degradation

Samples of heparan sulfate proteoglycan in 100 µl of water were treated with 20 µl of 3 M sodium nitrite and 20 µl of glacial acetic acid at room temperature for 80 min. Excess nitrous acid was destroyed by incubation with 50 µl of 3 M glycine for 1 h at room temperature. The products were analyzed on a Bio-Gel P-4 column.

Alkaline β-Elimination of Proteoglycans

The proteoglycan samples were treated for 20-24 h at 45°C with 0.1 M sodium hydroxide containing 1 M sodium borohydride [44]. The solution was cooled in an ice-bath and neutralized with 4 M acetic acid. After lyophilization, the β-eliminated material was chromatographed on Bio-Gel P-10 (0.9 × 100 cm) in 0.1 M pyridinium acetate, pH 5.2. The excluded material was analyzed on Sepharose CL-4B and CL-6B columns (0.9 × 106 cm) in 0.1 M pyridinium acetate, pH 5.0. The released *O*-linked oligosaccharides were analyzed by gel filtration on Bio-Gel P-6 (0.9 × 108 cm) in 0.1 M Tris-maleate, pH 6.0.

Hexosamine and Hexosaminitol Analysis

Samples were hydrolyzed for 18 h at 110°C with 6 M HCl under a nitrogen atmosphere. After evaporation under vacuum in a desiccator, the products were analyzed on a Beckman Model 120C amino acid analyzer. Buffer containing borate was used for analysis of hexosaminitols [53].

Radioactivity Measurements

Aliquots (0.2-1.0 ml) of aqueous solutions were mixed with ten volumes of ACS scintillati-

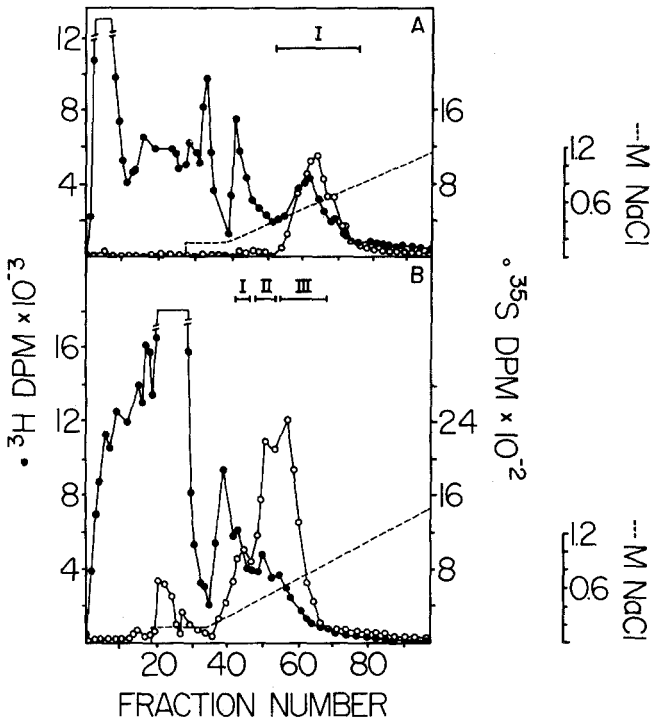

Figure 2. DEAE-Sephacel chromatography of plasma membrane extracts.
A, HBL cells; B, MDA cells. Plasma membrane extracts were applied to a DEAE-Sephacel column (0.8 × 8 cm) equilibrated with 50 mM sodium acetate, pH 6.0, containing 6 M urea and 0.5% CHAPS. The column was washed with 0.15 M sodium chloride in the above buffer and then eluted with a linear gradient of 0.15-1.2 M sodium chloride in the same buffer. Fractions of 2 ml were collected and aliquots were measured for radioactivity. Fractions were pooled as indicated by bars.

on mixture (Amersham) and the radioactivity quantified with an Intertechnique Model SL-4000 liquid scintillation spectrometer equipped with a dpm calculating module.

Results

Isolation of Plasma Membrane-associated Proteoglycans

Both normal (HBL-100) and malignant (MDA-MB-231) human breast cells synthesized ^{3}H- and ^{35}S-labeled proteoglycans. Of the total ^{35}S label incorporated, approximately 30-35% remained associated with cells after harvesting while the remaining 65-70% was recovered in the medium. Plasma membranes were prepared by an established procedure [34] which has been further tested in our laboratory for membrane isolation from

cultured cells (Murray MC, Bhavanandan VP, Davidson EA; unpublished results). The specific activities of 5'-nucleotidase and (Na^+, K^+)-ATPase were about 90 fold higher in the purified plasma membranes compared to the cell homogenate, indicating a high degree of purification. Only low levels of glucose-6-phosphatase (9%), *N*-acetylglucosaminidase (6%) and succinate dehydrogenase (10%) activities were observed compared to the cell homogenate, indicating 5-10% contamination of endoplasmic reticulum, lysosomes, mitochondria and possibly other organelles. In addition, a parallel membrane preparation was examined by electron microscopy. Several sections taken from the top to the bottom of the pellet confirmed the low level of contamination indicated by the enzymatic data. Approximately 55-60% (based on ^{35}S activity) of the cell-associated proteoglycans were recovered in the plasma membrane fractions. On DEAE-Sephacel chromatography of the plasma membrane extract from HBL cells, approximately 70% of the ^{3}H activity was recovered in the starting buffer and in buffer containing 0.15 M sodium chloride. No significant ^{35}S activity was found in this material, which was therefore presumed to contain glycoproteins and perhaps some glycolipids. Approximately 13% of the ^{3}H activity was eluted between 0.2 and 0.4 M sodium chloride. An aliquot of this fraction on hydrolysis and analysis for hexosamines showed 84% glucosamine and the reminder galactosamine. Since essentially no ^{35}S activity was found in this material, we considered the possibility that this might be mainly hyaluronic acid. However, on treatment with bacterial hyaluronidase (0.1 M sodium acetate, pH 5.0, for 24 h at 37°C) followed by gel filtration on Bio-Gel P-4, it was found to be undegraded. Treatment of this fraction with *Vibrio cholerae* sialidase or with mild acid (0.05 M sulfuric acid for 1 h at 80°C) followed by Bio-Gel P-4 chromatography showed that 25% of the ^{3}H activity co-eluted with reference *N*-acetylneuraminic acid. These results indicated that this fraction also contained glycoproteins.

About 17% of the ^{3}H activity and almost all of the ^{35}S activity was eluted as a broad peak between 0.4 and 0.85 M sodium chloride (Fig. 2A). This fraction was pooled and analysis of an aliquot for hexosamines showed 80% glucosamine and 20% galactosamine.

The extract of the plasma membranes of the MDA cells was chromatographed on a DEAE-Sephacel column (Fig. 2B). Approximately 84% of the ^{3}H activity was either unbound or eluted with buffer containing 0.15 M sodium chloride. Essentially no ^{35}S activity was found in this material which again was presumed to contain glycoconjugates other than proteoglycans. A relatively small proportion of the ^{3}H activity was unbound, whereas in the case of the HBL plasma membranes more ^{3}H activity was found in the unbound fraction than in the fraction eluted with 0.15 M sodium chloride (cf. Fig. 2A and 2B). These data suggest that more of the MDA plasma membrane glycoproteins and glycolipids are acidic compared with those of HBL plasma membranes, possibly reflecting a higher degree of sialylation of MDA glycoproteins.

About 7% of the ^{3}H activity was eluted between 0.15 and 0.4 M sodium chloride, and a small portion of the ^{35}S activity was also found in this fraction. This fraction was totally resistant to the action of bacterial hyaluronidase and approximately 23% of the ^{3}H activity was released as sialic acid on treatment with *Vibrio cholerae* sialidase or with mild acid. Therefore, it probably contains sialylated glycoproteins.

The balance of the ^{3}H activity (9%) and almost all of the ^{35}S activity was eluted between 0.4 and 0.85 M sodium chloride. Three distinct peaks of ^{35}S activity were evident, and the fractions were pooled as indicated in Fig. 2B. Aliquots of each fraction were hydrolyzed

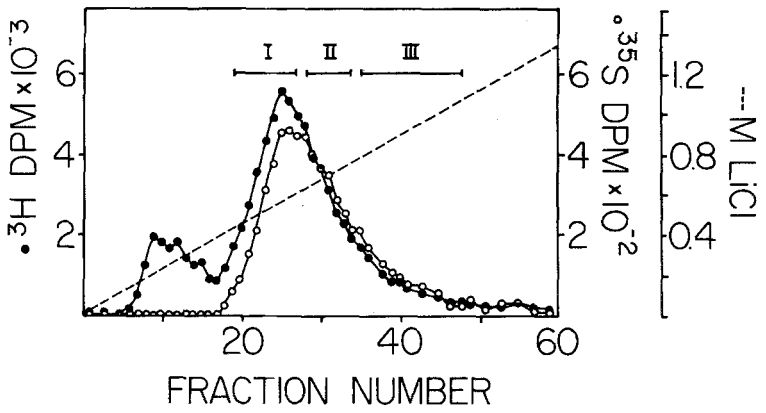

Figure 3. DEAE-Sepharose 6B chromatography of proteoglycans from plasma membranes of HBL cells. Sulfated components (fraction I, Fig. 2) were applied to a DEAE-Sepharose 6B column (0.8 × 10 cm) equilibrated with 50 mM Tris-HCl, pH 7.6, containing 6 M urea and 0.5% CHAPS. Elution was with a linear gradient of 0-1.5 M lithium chloride in the same buffer. Fractions were pooled as indicated by bars based on examination of the elution profile.

with acid and analyzed for hexosamines. Based on their hexosamine composition it appeared that peaks I and II consisted mainly of heparan sulfate proteoglycans and peak III mainly of chondroitin sulfate proteoglycan.

Dissociative cesium chloride density gradient centrifugation of the HBL plasma membrane proteoglycans (recovered after DEAE-Sephacel chromatography) indicated that both the ^{3}H and the ^{35}S activities were distributed throughout the gradient (data not shown). When samples from different regions of the gradient were analyzed for hexosamine composition, no marked differences were noted. Therefore this method was not considered suitable for further purification.

The HBL proteoglycan fraction and the three proteoglycan fractions from the MDA plasma membranes were separately fractionated on DEAE-Sepharose 6B using a lithium chloride gradient. The sulfated components from HBL plasma membranes, eluted as a broad peak without any clear separation (Fig. 3). However, when the fractions were pooled as indicated and analyzed for their hexosamine compositions (Table 1), it was found that fractions HPG-Ic and HPG-IIc were rich in glucosamine and fraction HPG-IIIc was rich in galactosamine.

DEAE-Sepharose 6B chromatography of MDA proteoglycan (peak I, Fig. 2B) using a lithium chloride gradient gave a distinct peak of only ^{3}H activity between 0.2 and 0.4 M lithium chloride and a peak with ^{3}H and ^{35}S activities between 0.4 and 0.6 M lithium chloride. Acid hydrolysis of the material from the former peak released 28% of the ^{3}H activity as material co-eluting with standard *N*-acetylneuraminic acid on Bio-Gel P-6. The peak material was totally resistant to the action of bacterial hyaluronidase and appeared to consist of sialylated glycoproteins. The hexosamine content of the latter peak (designated MPG-Ic) was almost exclusively glucosamine, suggesting that it was a heparan sulfate proteoglycan. Fraction II (Fig. 2B), on DEAE-Sepharose chromatography, gave a peak

Table 1. Hexosamine compositions of plasma membrane-associated proteoglycans.

Source	Fraction	Hexosamine composition (percent ^3H activity)	
		$GlcNH_2$	$GalNH_2$
HBL	HPG-Ic	95	5
	HPG-IIc	84	16
	HPG-IIIc	12	88
MDA	MPG-Ic[a]	93	7
	MPG-IIc	86	14
	MPG-IIIc	10	90

[a] Intracellular degradation products occurred as a contaminant in the isolated plasma membrane.

with only ^3H activity between 0.2 and 0.4 M lithium chloride; a distinct but not well-resolved component with both ^3H and ^{35}S activities between 0.4 and 0.6 M lithium chloride; and a major ^3H and ^{35}S activity peak between 0.6 and 0.8 M lithium chloride. The peak consisting only of ^3H activity appeared to contain sialoglycoproteins. The peak eluting between 0.4 and 0.6 M lithium chloride contained almost exclusively glucosamine and therefore was pooled with MPG-Ic. The major peak, between 0.6 and 0.8 M lithium chloride, also contained mainly glucosamine (86%) and was designated MPG-IIc. Fraction III (Fig. 2B), on DEAE-Sepharose chromatography, gave a broad peak between 0.8 and 1.2 M lithium chloride and a minor but not well resolved peak between 0.6 and 0.8 M lithium chloride; both contained ^3H and ^{35}S activities. The latter peak contained mainly glucosamine (87%) and therefore was pooled with MPG-IIc. However, the former peak (designated MPG-IIIc) contained predominantly galactosamine (90%) and thus appeared to be a chondroitin sulfate proteoglycan. The hexosamine compositions of the pooled fractions MPG-Ic, MPG-IIc and MPG-IIIc are summarized in Table 1.

Characterization of Proteoglycans

All six proteoglycan fractions (HPG-Ic, HPG-IIc, HPG-IIIc, MPG-Ic, MPG-IIc and MPG-IIIc) were chromatographed separately on a Sepharose CL-4B column (Fig. 4). Fraction HPG-Ic eluting as a single symmetrical peak with k_{av} 0.59 (M_r ~60 000) preceded a small amount of poorly-resolved higher molecular weight material (Fig. 4A). HPG-IIc was resolved into two peaks with k_{av} 0.36 (M_r ~400 000) and 0.60 (M_r ~50 000) (Fig. 4B). The latter peak had a hydrodynamic size comparable to that of HPG-Ic. Fraction HPB-IIIc eluted as a rather broad peak with k_{av} 0.36 (M_r ~400 000), plus a distinct, slowly eluting lower molecular weight species with k_{av} 0.60 (M_r ~50 000) (Fig. 4C).

Approximately 75% of the ^3H and ^{35}S activities of MPG-Ic eluted as a single symmetrical peak of k_{av} 0.83 (M_r ~8 500) and the remainder as a distinct but broad peak of higher molecular weight with k_{av} 0.57 (M_r ~80 000) (Fig. 4D). The elution pattern of MPG-IIc was more complex, with at least three distinct ^{35}S activity peaks k_{av} 0.24 (M_r ~700 000), 0.57 (M_r ~80 000), and 0.77 (M_r ~10 000) (Fig. 4E). This fraction, therefore, appeared to be very heterogeneous in size, ranging from a very high molecular weight species to those

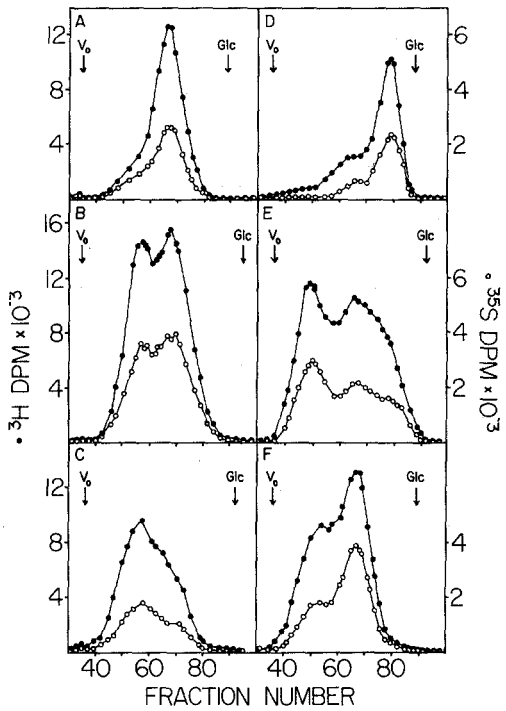

Figure 4. Sepharose CL-4B chromatography of proteoglycan fractions.
A, HPG-Ic; B, HPG-IIC; C, HPG-IIIc; D, MPG-Ic; E, MPG-IIc; and F, MPG-IIIc. Proteoglycan fractions obtained by DEAE-Sepharose 6B chromatography of plasma membrane proteoglycans were applied to a Sepharose CL-4B column (0.9 x 108 cm) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 4 M Gdn-HCl and 0.5% (w/v) Triton-X-100. Elution was with the same buffer. The elution positions of blue dextran (V_o) and glucose are indicated.

comparable with the major proteoglycans of MPG-Ic. Fraction MPG-IIIc gave two distinct peaks containing both ^{3}H and ^{35}S activities, with k_{av} 0.34 (M_r ~ 450 000) and 0.56 (M_r ~ 80 000) (Fig. 4F).

Nitrous Acid Degradation of Heparan Sulfate Proteoglycans

On nitrous acid treatment [41] followed by gel filtration on Bio-Gel P-4 (data not shown), about 90% and 80% of the ^{3}H activities from HPG-Ic and HPG-IIc, respectively, were eluted in the included volume (Table 2). Similar treatment of MPG-Ic and MPG-IIc resulted in approximately 90% and 80%, respectively, of the ^{3}H activity eluting in the included volume on Bio-Gel P-4 (Table 2). These results further indicated that HPG-Ic, HPG-IIc, MPG-Ic, and MPG-IIc were mainly heparan sulfate proteoglycans, in agreement with their hexosamine composition.

Table 2. Results of enzymatic and chemical degradations of proteoglycan fractions.

Fraction	Percent degraded[a]		
	Heparitinase	Nitrous acid	Chondroitinase ABC
HPG-Ic	85	91	NT[b]
HPG-IIc	82	82	NT
HPG-IIIc	NT	NT	90
MPG-Ic	84	90	NT
MPG-IIc	80	80	NT
MPG-IIIc	NT	NT	90

[a] Approximate percent distribution of ^{3}H activity in low molecular weight material as determined by Bio Gel P-4 chromotography.
[b] Not treated.

Heparitinase Degradation

Aliquots of HPG-Ic, HPG-IIc, MPG-Ic and MPG-IIc were treated with heparitinase [42] and the products analyzed on a Bio-Gel P-4 column. In each case, approximately 80 to 85% of the material was eluted in the included volume (Fig. 5 and Table 2), confirming the heparan sulfate nature of the major portion of these proteoglycan fractions.

Chondroitinase Degradation

Aliquots of HPG-IIIc and MPG-IIIc were treated with chondroitinase ABC [43], and the products chromatographed on a Bio-Gel P-4 column. About 90% of both the ^{3}H and ^{35}S activities were eluted in the disaccharide region with no significant amount in the higher molecular weight regions. The nature of the monosulfated disaccharides was examined by paper chromatography [43]. Approximately 83% and 87% of monosulfated disaccharides from HPG-IIIc and MPG-IIIc, respectively, were 4-sulfated, while the reminder was 6-sulfated (data not shown). Further, treatment of monosulfated disaccharides from HPG-IIIc and MPG-IIIc with chondro-4-sulfatase or chondro-6-sulfatase resulted in their quantitative conversion to ΔDi-OS (data not shown).

The proteoglycan fractions MPG-IIIc and HPG-IIIc were also treated with chondroitinase AC, and the products were analyzed on Bio-Gel P-4 (data not shown). About 80% of each proteoglycan fraction was degraded into unsaturated disaccharides. Some high molecular weight fragments were also observed, indicating the presence of some iduronic acid residues in the glycosaminoglycan chains. Since the resistant residues comprise less than 20% of the total ^{3}H activity, HPG-IIIc and MPG-IIIc were identified as primarily chondroitin sulfate proteoglycans.

WGA-Sepharose Affinity Chromatography

Aliquots of proteoglycan fractions were chromatographed on a WGA-Sepharose column using buffer containing 0.1% Triton X-100 [33]. Approximately 5-10% of the ^{3}H-activity of each of the fractions was bound to WGA and eluted with *N*-acetylglucosamine indicating the presence of contaminating glycoproteins.

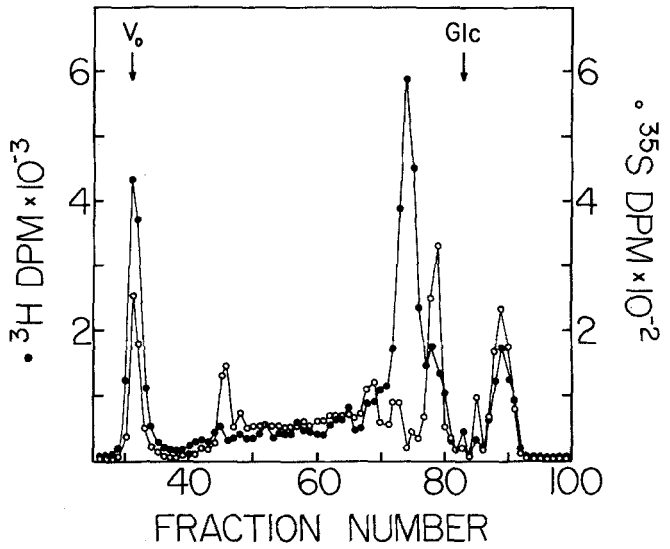

Figure 5. Bio-Gel P-4 chromatography of heparitinase-treated HPG-Ic.
An aliquot of proteoglycan fraction HPG-Ic was treated with heparitinase as described in the Materials and Methods section. The products were chromatographed on a Bio-Gel P-4 column (0.9 × 100 cm). Elution was with 0.1 M pyridine/acetic acid, pH 5.2. Similar elution profiles were obtained for heparitinase-treated HPG-IIc, MPG-Ic and MPG-IIc (not shown). The elution positions of a reference glycosaminoglycan (V_o) and glucose are indicated.

Alkaline β-Elimination and Analysis of the Released Glycosaminoglycans

Aliquots of proteoglycan fractions were subjected to alkaline β-elimination in the presence of sodium borohydride [44] to release the glycosaminoglycan chains. The products were chromatographed on a Bio-Gel P-10 column. The glycosaminoglycans obtained from all the proteoglycan fractions except MPG-Ic were excluded from Bio-Gel P-10. A significant amount of the glycosaminoglycan chains obtained from MPG-Ic was included on Bio-Gel P-10, indicating the presence of relatively lower molecular weight chains in this fraction.

The released glycosaminoglycan chains were recovered and further chromatographed on a Sepharose CL-6B column (Fig. 6). The heparan sulfate chains obtained from HPG-Ic and HPG-IIc were eluted as broad single symmetrical peaks with k_{av} 0.55 (M_r 14 000, Fig. 6A) and 0.42 (M_r 20 000, Fig. 6B), respectively. The heparan sulfate chains of MPG-Ic were eluted at k_{av} 0.75 (M_r 8 000) with a distinct but not well-separated peak in the higher molecular weight region (k_{av} 0.60, M_r 12 000, Fig. 6D). The heparan sulfate chains of MPG-IIc were eluted as two broad unresolved peaks with k_{av} 0.40 (M_r 21 000) and 0.66 (M_r 8 500, Fig. 6E). It is apparent from their elution behaviors that a portion of the heparan sulfate chains derived from MDA plasma membrane proteoglycans (particularly those from MPG-Ic) are much smaller than, but the reminder are comparable in size to, those from HBL plasma membrane. Additionally, the heparan sulfate chains from MDA are more heterogeneous in size than those derived from HBL.

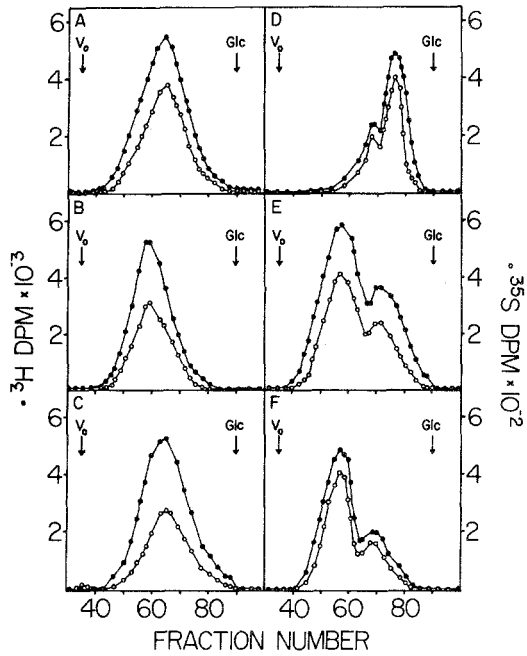

Figure 6. Sepharose CL-6B chromatography of glycoaminoglycans released by alkaline β-elimination of proteoglycans.
A, from HPG-Ic; B, from HPG-IIc; C, from HPG-IIIc; D, from MPG-Ic; E, from MPG-IIc; and F, from MPG-IIIc. Samples were applied to a Sepharose CL-6B column (0.9 × 106 cm) and the column eluted with 0.1 M pyridine/acetic acid, pH 5.2. The elution positions of blue dextran (V_o) and glucose are indicated.

The chondroitin sulfate chains of HPB-IIIc proteoglycans were eluted on Sepharose CL-6B as a single broad peak with k_{av} 0.55 (M_r 14 000, Fig. 6C), whereas those from MPG-IIIc were eluted as two distinct but not well-resolved peaks with k_{av} 0.40 (M_r 21 000) and 0.62 (M_r 11 000, Fig. 6F).

Discussion

In a previous paper [33], the proteoglycans secreted into the culture medium by a malignant human breast cell line (MDA-MB-231) were characterized and compared with the corresponding proteoglycans from a normal human breast cell line (HBL-100). The results presented in this paper demonstrate that the cell membrane-associated proteoglycans of MDA-MB-231 cell line differ significantly in their charge density, hydrodynamic size, and glycosaminoglycan compositions from those of the HBL-100 cell line.

Both MDA-MB-231 and HBL-100 cell lines synthesized $^3H/^{35}S$-labeled proteoglycans during growth in medium containing [^{3}H]-glucosamine and [^{35}S]Na_2SO_4. Approximately 30-35% of the total $^3H/^{35}S$-labeled proteoglycans produced by these cell lines was found to be associated with the cell layer, and 55-60% of this was present in isolated plasma membranes. The latter proteoglycans could be extracted effectively with 4 M guanidine hydrochloride in the presence of Triton X-100. Extraction and subsequent ion-exchange chromatography of the plasma membrane extracts were performed in the presence of

protease inhibitors to prevent the degradation of the protein cores of proteoglycans. During ion-exchange chromatography on DEAE-Sephacel, Triton X-100 was replaced by CHAPS, as this zwitterionic detergent is dialyzable [23]. Ion-exchange chromatography of plasma membrane extracts separated the bulk of the glycoproteins from the proteoglycans.

The plasma membranes of both cell lines contained heparan sulfate and chondroitin sulfate proteoglycans. There was a significant quantitative difference in these two classes of proteoglycans from the plasma membranes of the MDA cell line compared with those of HBL cell line. The HBL plasma membranes contained a higher proportion of heparan sulfate proteoglycan compared to the MDA plasma membrane. These observations are in agreement with previous findings that normal cells contain higher proportions of cell membrane-associated heparan sulfate proteoglycans [1]. In contrast, increased amounts of chondroitin sulfate proteoglycan were found to be present in tumor cell lines [6, 13-15]. It is interesting to note that even though a significant difference in cell surface proteoglycan composition exists in these cell lines, noticeable changes were not observed for the proteoglycans secreted by these cell lines into the culture medium. In fact, the culture medium of both cell lines contained nearly the same amounts of heparan sulfate (20%) and chondroitin sulfate (80%) proteoglycans [33].

The plasma membranes of MDA cells contained two distinct heparan sulfate proteoglycan populations with $M_r \sim 700\,000$ and $M_r \sim 80\,000$. The proteoglycan species are comparable in their molecular size to two heparan sulfate proteoglycan species secreted into the medium by MDA cells [33]. The lower charge density heparan sulfate proteoglycan species with a significantly smaller hydrodynamic size (k_{av} 0.83, $M_r \sim 8\,500$) accounts for approximately 10% of the total proteoglycans from MDA plasma membrane. They seem to contain a single glycosaminoglycan chain either free or bound to small peptides, as no noticeable change in their size was observed after β-elimination (see later).

Low molecular weight proteoglycan species with a single glycosaminoglycan chain have been shown to be present as intracellular degradation products in other systems [23, 46-49]. The low molecular weight heparan sulfate had a molecular weight similar to that of a heparan sulfate proteoglycan species isolated by Chang *et al.* from smooth muscle cells [48] and by Hascall and Yanagishita from rat ovarian granulosa cells [23]. This latter product was shown to arise due to intracellular degradation. Therefore, it is possible that the low molecular weight heparan sulfate proteoglycan from MDA cells may also be the result of intracellular degradation.

The HBL plasma membrane contained two hydrodynamic size heparan sulfate species with k_{av} 0.36 and 0.60. The larger species has a slightly lower molecular weight compared to the heparan sulfate-proteoglycan secreted into the medium by HBL cells [33]. Species of molecular size comparable to that of the smaller hydrodynamic size species were not found in the medium.

The chondroitin sulfate proteoglycans from the plasma membranes of both cell lines contained at least two hydrodynamic size species, not well resolved by gel filtration. However, those from MDA plasma membrane contained a much greater proportion of the smaller species than those from the HBL membranes. The chondroitin sulfate proteoglycans of both cell lines were predominantly 4-sulfated with a small proportion of 6-sulfate.

The low charge density heparan sulfate proteglycans from MDA cells contained short glycosaminoglycan chains (M_r 8 000). No noticeable change in their molecular size has been observed even after alkaline β-elimination suggesting that these species contain little of the protein core although the presence of some peptide is possible. The heparan sulfate proteoglycans from MDA plasma membrane contained two glycosaminoglycan chain types with M_r 21 000 and M_r 8 500, and have a broad molecular weight distribution. Presumably some of the glycosaminoglan chains have nearly the same molecular size as those observed in case of secreted proteoglycans [33], but are more heterogeneous in size.

Even though the major chondroitin sulfate proteoglycans of MDA plasma membranes were smaller in size than those from HBL plasma membrane, the major portion of the glycosaminoglycans of the former are bigger (M_r 21 000) than those from the latter (M_r ~14 000). Further, the MDA plasma membrane chondroitin sulfate contained distinctive low molecular weight glycosaminoglycan chains while molecules of comparable size were absent in the chondroitin sulfate proteoglycans from HBL plasma membrane.

WGA-Sepharose affinity chromatography of various proteoglycan fractions from plasma membranes of both cell lines indicated that all the fractions contained 5-10% of their ^3H activity as sialylated glycoproteins. Preliminary experiments suggested that these highly sialylated glycoproteins from the plasma membranes tend to be strongly associated with proteoglycans and rather extensive purification steps are needed to resolve them.

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