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Glycosaminoglycans of the Plasma Membranes of Renal Tubule and Liver Cells

Dora Lis and Benito Monis

Primera Cátedra de Histología e Instituto de Biología Celular, Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Casilla de Correo 220, 5,000 Córdoba, Argentina

Glycosaminoglycans were isolated from plasma membranes of hepatic and renal tubule cells of guinea pig. Plasmalemma of renal tubule cells contained more total glycosaminoglycans, hyaluronic acid, chondroitin-4 sulfates and chondroitin-6 sulfates, and less dermatan sulfates and heparin sulfates than liver plasma membranes. These glycocalyx components, owing to their polyanionic properties, may have a role in the transport of water, ions, and macromolecules across the cell membrane.

Key words: glycosaminoglycans, glycocalyx, liver and kidney plasma membranes, hyaluronic acid, chondroitin sulfates, heparin sulfates

It has been shown that many plasma membranes contain glycoproteins and glycolipids [1]. Yet there are less data on glycosaminoglycans (GAGs) in cell membranes. Thus, various GAGs were identified by nondisruptive procedures on the surface of normal cells in vitro, such as L929 and Chinese hamster ovary (CHO) fibroblasts [2–4], and endothelium of rabbit aorta [5] and rabbit lung [6], as well as in transformed and tumoral cells: HeLa [2,6], HEp 2 [6], rat ascites hepatoma [7], BHK21/C13, and mouse 3T3 fibroblasts [8–10]. Nevertheless, most of these reports gave no quantitative data nor was the pattern of membrane-bound GAGs established. The latter were also found in isolated plasma membranes of liver and hepatoma cells of rat [7,11–13], and in the fat globule membrane of cow milk [14]. A proteoheparin sulfate has been isolated from plasma membranes of an ascites hepatoma [15]. Here we report the composition of GAGs obtained from plasma membranes of kidney and liver cells of guinea pig.

EXPERIMENTAL

Separation of Plasma Membranes

Plasma membranes were isolated from livers and kidneys of 100 guinea pigs. Animals were decapitated, bled, and perfused through the left ventricle with cold 0.9% NaCl. Liver

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and kidneys were immediately excised. Renal tubules were separated from the kidney medulla by digestion with 0.05% collagenase (EC 3.4.24.3) in 10 mM sodium phosphate buffer, pH 7.4, containing 126 mM NaCl/5mM KCL/1.2 mM $MgSO_4$/1 mM $CaCl_2$/10 mM sodium acetate, for 3 h at room temperature [16,17]. Plasma membranes were obtained by hyposmotic lysis in 5 mM EDTA at 4°C and differential centrifugation [18]. Liver plasma membranes were isolated by homogenizing tissue slices in 0.3 M sucrose at 4°C followed by differential centrifugation and purification in a sucrose gradient [19]. Membranes were also obtained by this procedure after separating the liver cells by a collagenase treatment. The purity of the membrane preparations was controlled by electron microscopy and enzymatic activities.

Electron Microscopy

Pellets were fixed overnight, at 4°C in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After rinsing in the buffer, pellets were refixed in 0.6% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, for 2 h at 4°C. Samples were rinsed with this buffer and taken through a series of graded alcohols and embedded in Epon 812. Thin sections were stained with lead citrate and uranyl acetate and observed in a electron microscope.

Enzymatic Markers

Aliquots of the tissue homogenates and membrane preparations which contained 0.2–0.4 mg of proteins were assayed for alkaline phosphatase (EC 3.1.3.1), 5′-nucleotidase (EC 3.1.3.5), glucose-6-phosphatase (EC 3.1.3.9) acid phosphatase (EC 3.1.3.2), and succinate dehydrogenase (EC 1.3.99.1). Blanks consisted of the enzyme source and all reagents, except the substrate.

Alkaline phosphate activity. Preparations were incubated with 10 mM β-glycerophosphate in 0.1 M TRIS – HCl buffer, pH 10.0/10 mM $MgCl_2$ for 20 min at 37°C [45].

5′-Nucleotidase activity was determined by incubating the samples with 2.4 mM adenosine-5′-phosphate in 0.08 M glycine buffer, pH 8.5/8 mM $MgCl_2$ for 15 min at 37°C [20].

Glucose-6-phosphatase activity. Preparations were incubated with 20 mM glucose-6-phosphate in 0.2 M sodium maleate buffer, pH 6.5, for 15 min at 37°C [21]. At the end of the reaction, trichloroacetic acid, final concentration 5% (w/v), was added in the cold, and materials were centrifuged at 7,500g for 15 min at 4°C. Inorganic phosphate was determined in the supernatant by a modification of the Fiske-SubbaRow method [22].

Acid phosphatase activity. Preparations were incubated with 5.5 mM p-nitrophenyl phosphate in 0.05 M sodium citrate buffer, pH 4.9. One volume of 0.04 M NaOH was added. Released p-nitrophenol was determined at 400 nm [23].

Succinate dehydrogenase activity was determined with 60 mM sodium succinate and 7.5 mM K_3 [$Fe(CN)_6$] in 0.15 M sodium phosphate buffer, pH 7.6/1.5 mM EDTA/1.5 M KCN/0.15% (w/v) bovine serum albumin. The change in absorbance was recorded at 455 nm during the first 2 min [24].

Isolation of Glycosaminoglycans

Membranes were defatted with 19 volumes of chloroform-methanol (2:1 v/v), digested at 65°C for 6 h with 100 mg/g wet weight of papain (EC 3.4.22.2) in 0.1 M sodium phosphate buffer, pH 6.5/5 mM EDTA/5 mM cysteine-HCl, treated with cold trichloroacetic acid (10% w/v, final concentration), neutralized with saturated NaOH, concentrated at 65°C, and chromatographed on Sephadex G-50 (column size 2.5 × 30 cm) eluting with distilled water. Excluded materials were dried, digested with 1 mg ribonuclease (EC 3.1.4.23) in 50

mM sodium phosphate buffer, pH 7.4/5 mM $MgCl_2$ for 3 h at 37°C, and rechromatographed on Sephadex G-50. Crude preparations thus obtained were fractionated on DEAE-Sephadex A-25 (0.25 × 20 cm). Fractions which eluted with 0.1 and 0.5 M NaCl, and 1.0, 1.5, 2.0, and 2.5 M NaCl in 0.01 M HCl, were salted out on Sephadex G-25 and concentrated [14,17].

Analytical Assays

Uronic acids [25], hexosamines [26], sulfates [27], and proteins [28] were determined in duplicate on the crude preparations and DEAE-Sephadex A-25 fractions.

Electrophoresis on cellulose acetate was carried out in 0.1 M barium acetate, pH 6.6, and 0.1 M HCl, pH 1.2 [29,30]. Electrophoretograms were stained with 1% (w/v) alcian blue, in 3% acetic acid, pH 2.5, and 0.05% toluidine blue in 60% (v/v) ethanol, for 30 min, and scanned in densitometer at 511 nm.

Enzymatic Assays

Aliquots of 0.5 M NaCl fraction were digested with leech hyaluronate lyase (EC 4.2.2.1) in 0.1 M sodium phosphate buffer, pH 5.6, for 3 h at 37°C [31], and electrophoresed on cellulose acetate.

Aliquots of 1.0–2.5 M NaCl fractions were subjected to digestion with chondroitin ABC and AC lyases (EC 4.2.2.4 and 4.2.2.5) in 0.25 M TRIS-HC-HCl buffer, pH 8.0/030 M sodium acetate/0.25 M NaCl for 6 h at 37°C, and chromatographed on Whatman No. 1 paper in butan-1-ol/acetic acid/1.0 M ammonia (2:3:1, v/v) for 15 h at room temperature. Ultraviolet-absorbing areas were cut and eluted with 0.01 M HCl. On the eluates, the absorbance at 232 nm was measured to determine the corresponding unsaturated disaccharides of chondroitin-4, chondroitin-6, and dermatan sulfates [32]. Nondigested materials present in the chromatogram origin were eluted with water and electrophoresed as above. Heparin sulfate content was calculated from the absorbance percentage of the electrophoretograms stained with toluidine blue.

Chemicals

Standards. Hyaluronic acid (potassium salt), chondroitin-4 sulfate, chondroitin-6 sulfate, and dermatan sulfate (sodium salts) and bovine serum albumin were obtained from Sigma Chemical Co. (St. Louis), heparin from Fisher Scientific Co. (Fair Lawn, New Jersey), and glucuronic acid and glucosamine-HCl from California Corporation for Biochemical Res. (Los Angeles).

Enzymes. Collagenase (Type I) was purchased from Sigma Chemical Co., papain from General Biochemicals (Chagrin Falls, Ohio), ribonuclease from BDH Chemicals (Poole, England), and leech hyaluronate lyase from Biotrics, Inc. (Boston). Chondroitin AC and ABC lyases and standard disaccharide kits were obtained from Miles Laboratories, Inc. (Elkhart, Indiana). All other reagents were analytical grade.

RESULTS

Electron microscopy revealed rather pure plasmalemma preparations devoid of other organelle contaminants, in keeping with the data on enzymatic activities found in homogenates of renal tubule and liver tissues and their corresponding membrane preparations (Table I.).

Plasma membranes of liver and renal tubule cells of guinea pig contained GAGs. Levels of uronic acids, hexosamines, sulfates, and proteins were higher in renal tubule cell

TABLE I. Enzyme activities of the Plasma Membrane Preparations of Renal Tubule and Liver Cells of the Guinea Pig

Enzyme	Renal tubule			Liver		
	HOM[a]	PM[a]	PM/HOM	HOM[a]	PM[a]	PM/HOM
Alkaline phosphatase	148.2	149.7	1.01	54.2	317.2	5.85
5′ -nucleotidase	197.5	165.0	0.84	51.2	604.2	11.80
Glucose-6-phosphatase	123.3	0	0	24.5	0	0
Acid phosphatase	1.5	1.0	0.70[b]	0.8	0	0
Succinate dehydrogenase	6.8	0	0	7.5	0	0

Renal tubules were lysed in 5 mM EDTA at 4°C for 5–10 min and centrifuged 5 min at 300g at 4°C. This was repeated four times. Then 0.25 volumes of 52.5% (w/v) sucrose in 20 mM Tris-HCl buffer, pH 7.4, was added to the supernatants. Materials were centrifuged at 7,000g for 10 min. The supernatants were mixed with 1 vol of 2.5% (w/v) Ficoll in 10 mM-Tris-HCl buffer, pH 7.6/50 mM glycerol/2 mM $MgCl_2$ and centrifuged at 11,000 for 10 min at 4°C. The pellet contained the plasma membranes. Liver slices were homogenized twice in 0.3 M sucrose at 4°C and centrifuged at 2,400g, 15 min. The pellet (nuclear fraction) was washed with 0.9% (w/v) NaCl and recentrifuged. Plasma membranes were obtained by washing this fraction three times with 0.9% NaCl/2 mM EDTA and centrifuging at 350g for 10 min. Membranes were pelleted at 70,000g for 15 min and purified in a sucrose gradient (densities: 1.16, 1.18, 1.20, and 1.22). After centrifuging at 53,000g, 75 min, interphase 1.16–1.18 was separated, diluted with one volume of distilled water, and centrifuged at 70,000g for 20 min. Proteins and enzyme activities were determined in aliquots of tissue homogenates (HOM) and plasma membrane preparations (PM), as described in Experimental.

[a]nkat/mg protein.

[b]Total enzyme activity in the plasma membrane preparation was 1.9% of that in the homogenate.

membranes (Table II). Results for liver plasma membranes obtained from homogenates of slices as well as cells separated with collagenase were similar. Uronic acids in the renal membrane preparations were concentrated thrice in respect to the cell homogenate (Table II, see also Lis and Monis [17]).

Chromatography of the crude preparations of liver and kidney plasma membranes on DEAE-Sephadex A-25 yielded six fractions (Tables III and IV). The first, which eluted with 0.1 M NaCl, was devoid of uronic acids and contained neutral and acid glycopeptides. The 0.5 M fractions accounted for 38% and 54% of the total uronic acids of the plasma membranes of liver and renal tubule cells, respectively, and corresponded to hyaluronic acid (Tables III–V). Renal tubule cell membranes contained a compound similar to that found in tubule preparations, which was identified as a keratan sulfate-like glycoprotein [17]. Sulfated GAGs eluted in the remaining four fractions, being those of renal tubule cell membranes of a higher degree of sulfation, as shown by the elution pattern on DEAE-Sephadex and the electrophoretic mobility in 0.1 M HCl (Tables III and IV). Densitometry of the electrophoretograms as well as enzymatic digestions demonstrated differences in the composition of sulfated glycosaminoglycans. Thus, dermatan sulfates represented about 25% of the sulfated GAGs in liver membranes but only 6% in the renal tubule plasmalemma. On the other hand, chondroitin-4 sulfates accounted for 55% of the uronic acids of the sulfated GAGs in the renal tubule cell membranes and 37% in the liver membrane preparation. Renal tubule membranes also contained less heparin sulfates and more chondroitin-6 sulfates (Table V).

TABLE II. Composition (μg/100 mg dried membrane) of the Crude Preparations of Plasma Membrane (PM) of Renal Tubule and Liver Cells of the Guinea Pig

Component	Renal tubule PM	Liver PM
Uronic acids	97.1	72.9
Hexosamines	261.2	224.5
Sulfates	31.0	12.3
Proteins	670	140

Crude preparations were isolated from defatted plasma membranes by digestion with papain and ribonuclease, precipitation with trichloroacetic acid, and chromatography on Sephadex G-50 (See Experimental); 92% of the GAGs added as internal standards was recovered by this procedure.

TABLE III. Composition (percentage of the glycosaminoglycan component recovered) of the DEAE-Sephadex A-25 Fractions of Plasma Membranes of Renal Tubule (RT) and Liver (L) Cells

M NaCl:	0.1		0.5		1.0		1.5		2.0		2.5	
Component	RT	L	RT	L	RT	L	RT	L	RT	L	RT	L
Uronic acids	0	0	54	38	8	15	13	22	10	20	15	5
Hexosamines	63	49	23	30	2	5	6	7	4	6	2	3
Sulfates	0	0	20	19	28	30	23	26	15	13	14	12
Proteins	84	40	16	20	0	7	0	6	0	20	0	7

Crude preparations were separated on DEAE-Sephadex A-25 by stepwise elution with NaCl solutions. Fractions were salted-out on Sephadex G-25 and concentrated at 65°C.

DISCUSSION

The present data indicate that GAGs are bound to the plasma membranes of liver and renal tubule cells of the guinea pig. Furthermore, all GAGs present in connective tissues were also identified in the two membranes herein studied. Differences in the absolute and relative concentrations of GAGs in both plasma membranes were observed. Thus, those of renal tubule cells contained more hyaluronic acid, chondroitin-4 and chondroitin-6 sulfates than liver cell membranes which were richer in dermatan and heparin sulfates (Table V).

Results also indicate that species-dependent differences in the pattern of GAGs of the plasma membrane of a given cell type may be even greater than those found in the plasmalemma of various cells of the same species. For example, cell membranes of rat renal tubules contained heparin sulfate (64.9 μg uronic acid/100 mg dry weight), the remaining being dermatan sulfates (5.3 μg) and hyaluronic acid (11.9 μg) (Lis and Monis, unpublished; compare with Table V). It has been shown [7,11] that plasma membranes of normal rat liver contained only heparin sulphate, which was also considered the only or the main constituent of various cell surfaces such as those of rat hepatoma [7,11–13], endothelium of rabbit aorta [5], and CHO and BHK 21/C13 fibroblasts [3,8]. The fat globule membrane of cow milk contained mainly hyaluronic acid and minor amounts of sulfated GAGs [14].

TABLE IV. Electrophoretic Mobilities of DEAE-Sephadex A-25 Fraction Components of Plasma Membranes of Renal Tubule (RT) and Liver (L) Cells

Buffer	Standards		0.5		1.0		1.5		2.0		2.5	
			RT	L	RT	L	RT	L	RT	L	RT	L
Barium acetate	Chondroitin-4/-6 sulfates	1.0	0.71	0.72	0.97	1.07	0.98	1.00	1.00	1.05	1.00	1.04
	Dermatan sulfates	0.73–0.78	0.63		0.73	0.79	0.73	0.76	0.71	0.76	0.04	0.80
	Hyaluronic acid	0.73–0.78			0.03	0.03	0.03	0.04	0.03	0.04		0.04
	Heparin	0.03–0.04										
HCl	Chondroitin-4/-6 sulfates	1.0	0.66	0.60	0.90	0.82	1.10	0.95	1.20	1.05	1.27	1.15
	Dermatan sulfates	1.0	0.75									
	Hyaluronic acid	0.60–0.66										
	Heparin	1.13–1.18										

Electrophoreses were carried out on cellulose acetate strips in 0.1 M barium acetate, pH 6.6, or in 0.1 M HCl, pH 1.2, at 3 V/cm for 3 h. Electrophoretograms were dried at 105° ± 5°C, 10 min, stained with 1% alcian blue, pH 2.5, and 0.05% toluidine blue in 60% (v/v) ehtanol for 30 min, and washed with ethanol (3 times, for 10 min). Strips stained with Alcian blue were cleared with methanol/acetic acid/glycerol (85:14:1, v/v). Mobilities were expressed in respect to chondroitin-4 and chondroitin-6 sulfates. Components of the 0.1 M fraction did not migrate in any of the buffers stained with the PAS and Coomassie brilliant blue but not with Alcian blue and toluidine blue. The 0.5 M fraction component was digested with leech hyaluronate-lyase. The 1.0–2.5 M fraction constituents migrating like chondroitin sulfates were digested by chondroitin AC and ABC lyases, whereas those with mobilities of 0.73–0.80 were digested only by chondroitin ABC lyase.

TABLE V. Glycosaminoglycans (μg uronic acid/100 mg dried membrane) of Renal Tubule and Liver Cell Membranes of the Guinea Pig

Glycosaminoglycan	Renal tubule PM	Liver PM
Hyaluronic acid	52.1	27.5
Chondroitin-4 sulfates	25.3	16.8
Chondroitin-6 sulfates	10.2	8.8
Dermatan sulfates	2.9	11.7
Heparin sulfates	6.6	8.2

Concentration of hyaluronic acid was determined by assaying the uronic acid content of the 0.5 M fraction. Sulfated GAGs were determined by adding the uronic acid content of fractions 1.0–2.5 M. Samples of 1.0–2.5 M fractions were digested with chondroitin ABC and AC lyases as described in Experimental. Chondroitin-4 and chondroitin-6 sulfates were calculated by determining the amount of unsaturated disaccharides engendered by chondroitin AC lyase digestion. Dermatan sulfates were calculated as the difference between the amount of unsaturated disaccharides produced by chondroitinase ABC and chondroitin AC lyases. Heparin sulfates were determined by densitometry of the barium acetate electrophoretograms stained with toluidine blue.

The reported data would indicate that GAGs are bound to plasma membranes of cells derived from any of the three germinal layers.

Differences in the pattern of GAGs in transformed and tumoral cells and during the cell cycle have been reported. Thus, a decrease in heparin sulfate and an increase in hyaluronic acid released by trypsin have been observed in polyoma and sarcoma virus-transformed fibroblasts [8,10,33]. The release of heparin sulfate from the cell surface of CHO fibroblasts varied during the cell cycle, being higher in the premitotic phase [4]. Resting 3T3 fibroblasts contained more surface heparin sulfate and less hyaluronic acid than their growing counterparts [10]. Relative concentrations of cell surface chondroitin and heparin sulfates varied in several ascites hepatomas [7,13].

The functions of the membrane-bound GAGs are unknown. Indeed, the biologic significance of these glycoconjugates, even in connective tissues, remains to be determined. GAGs are highly negatively charged electrolytes due to the presence of carboxyl and sulfate groups. Their polyanionic characteristics are probably important in determining their biologic functions, perhaps by changing some physicochemical properties of the cell environment. These molecules, mainly chondroitin and heparin sulfates interact strongly with cations [34,35] and polycations, such as proteins, dyes, etc. [36, 37], while undergoing conformational changes [38,39], whereas hyaluronic acid may retain water [40]. They also markedly affect the diffusion of ions and neutral and electrically charged macromolecules [36,41].

All GAGs are compounds of high molecular weight which have a filamentous appearance [42]. This may account, in part, for the ultrastructural organization of glycocalyces which are made up of a feltwork of filamentous and/or granular components extending away from the plasma membrane itself [43, 44] and forming intricate structures which might regulate the cell communication with its milieu. A role of these glycoconjugates in transport across membranes can thus be suggested. GAGs seem also to be involved in processes of morphogenesis, cell division, and differentiation, as well as cellular interactions [4,6,31].

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