New role of glycosaminoglycans on the plasma membrane proposed by their interaction with phosphatidylcholine

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Abstract Glycosaminoglycan side chains of membrane proteoglycans have been claimed to be located at the outermost layer of the glycocalyx surrounding the cell. In this study measurements by surface plasmon resonance and solid-phase assay have shown that both chondroitin sulfate and keratan sulfate but not heparin associate with phosphatidylcholine under physiological conditions. Spectrophotometric measurements also showed that chondroitin sulfate restricts the lateral diffusion of phosphatidylcholine in liposomes. These findings indicate that chondroitin sulfate and/or keratan sulfate chains of membrane proteoglycans crouch on the surface of the membrane while heparan sulfate chains stretch outward from the membrane surface as postulated traditionally.

Key words: Glycosaminoglycan; Phospholipid; Membrane; Microdomain

1. Introduction

The elucidation of membrane organization is of prime importance for the understanding of its functions. Increasingly sophisticated membrane models have been proposed. In 1971, the fluid mosaic model emphasized the autonomy and diffusional mobility of membrane lipids and proteins and the consequent lack of lateral organization of the bilayer membrane [1]. Subsequently, attention focused on the elucidation of the asymmetrical organization of the membrane molecules and the cell surface organization which determines a face of the cell [2–4]. A number of experimental results indicate that the outer leaflet of the membrane is a mixture of various lipid molecules organized and segregated in microdomains, although it is mainly composed of phosphatidylcholine (reviewed by Edidin and Jacobson et al. [5,6]). Recently functional microdomains such as rafts in cell membranes were proposed [7]. The carbohydrate coat of the cell named glycocalyx is composed of three kinds of glycoconjugates, that is, glycolipids, glycoproteins and proteoglycans which can be likened to grasses, bushes and tall trees in reference to their respective heights from the ground (cell surface).

Recently, special attention has been given to the role of membrane-bound proteoglycans. They impose structural constraints, function as growth-supportive or -suppressive molecules, possess adhesive and anti-adhesive properties, act as major biological filters, promote angiogenesis, induce neurite outgrowth and deliver growth factors to target cells during normal development and in pathological states (for reviews, see [8,9]). A number of proteoglycans have been characterized, based on the type of their glycosaminoglycan chains and their location along the polypeptide backbone. The glycosaminoglycan chains vary greatly in number and type depending on the proteoglycans. In most membrane proteoglycans chondroitin sulfate and keratan sulfate chains uniquely attach to the nearby cell surface, although the biological meanings are unknown.

Most of the known proteoglycan binding molecules recognize the glycosaminoglycan portions of the proteoglycans. On the other hand, certain proteoglycans express C-type lectin domain(s) by which they can combine with the carbohydrate chains of other biological molecules (for review, see [8]). Krumbiegel and Arnold [10] have found by microelectrophoresis measurements with liposomes that heparin and its homologue, dextran sulfate, bind phosphatidylcholine, although under non-physiological conditions. Using different assay systems, we demonstrate here that under physiological conditions chondroitin sulfate and keratan sulfate, but not heparin, bind phosphatidylcholine.

2. Materials and methods

2.1. Polymers

Heparin was purchased from Wako (Osaka, Japan; Lot ACP1104), heparan sulfate (Lot S96301), chondroitin (Lot S95301), chondroitin sulfate A (Lot S93401), chondroitin sulfate C (Lot S92Y06) and dermatan sulfate were from Seikagaku (Tokyo, Japan). Chondroitin sulfate, a mixture of ca. 60% chondroitin sulfate A and ca. 40% chondroitin sulfate C, was prepared from bovine bronchial tubes by previously reported methods [11]. Keratan sulfate was prepared from shark fin according to the method previously reported [12]. Fucoidan (Lot 174H3862), dextran sulfate (Lot 26H0133) and polygalacturonic acid (Lot 115H3776) were from Sigma.

2.2. DAWN analysis

DAWN analysis, which is also called SEC-MALL (aqueous size exclusion chromatography and multiangle laser light scattering), was performed according to the method reported by Nagy [13]. The size exclusion chromatography consisted of a Shodex size exclusion column Protein KW-803, Shodex RI-71 refractive index detector (Showa Denko, Tokyo, Japan) and a Wyatt Technology Model MINI DAWN multiangle laser light scattering photometer (Wyatt, Santa Barbara, CA, USA) and was operated at room temperature with a flow rate of 1.0 ml/min. Molecular weights were calculated using ASTRA software from Wyatt Technology. Specific refractive index increment values (dn/dc) were used, in the case of protein 0.180 ml/g, in the case of glycosaminoglycan 0.129 ml/g.

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2.3. Biacore analysis

Dimyristoylphosphatidylcholine (PC), phosphatidylethanolamine (PE, egg yolk), phosphatidylserine (PS, bovine brain) and phosphatidylinositol (PI, bovine liver) were from Sigma. Liposomes composed of PC alone and PS/PC, PE/PC and PI/PC in a ratio of 1:1 were prepared by sonication and filtration. The hydrophobic chip HPA was initially washed with 0.1 mM β-octyl-D-glucoside for 5 min at a flow rate of 5 μl/min. Liposomes in 10 mM HEPES-buffered saline (pH 7.4) (HBS) were then injected for 30 min at a flow rate of 1 μl/min. An increase of the flow rate to 5 μl/min as well as a brief injection of 10 mM NaOH was sufficient to wash off the unbound phospholipids and to plane the phospholipid surfaces. The chip had phospholipid mixtures immobilized (ca. 2000 RU). 40 μl of glycosaminoglycan solution in HBS was then injected in the presence of 1 mM calcium ion at a flow rate of 20 μl/min. The chip was regenerated after each cycle of measurements by injecting 10 μl of 0.5 M EDTA. The kinetic parameters were calculated by the evaluation program BIAevaluation 3.0.

2.4. Binding assay of PC and chondroitin sulfate using ELISA plates

Oxidation of chondroitin sulfate was performed at room temperature in 0.1 M sodium acetate (pH 5.5) containing 3.3 mM sodium metaperiodate in the dark. After 1 h incubation, the oxidation was stopped by adding an excess of sodium sulfite. The chondroitin sulfate was further incubated with biotin-hydrazide (Pierce) at a final concentration of 0.3 mg/ml. The excess biotin was then removed on an NAP-5 column (Pharmacia). Phospholipid-coated ELISA plates were used. 50 μl of 1 mg/ml of phospholipids in ethanol was added to the well of the ELISA plate, and then allowed to stand for 18 h at 37°C. The phospholipid-coated well was washed with HBS and blocked with 1% bovine serum albumin for 1 h, and then 50 μl of biotin-labeled chondroitin sulfate was added in the presence of 1 mM $CaCl_2$. The well was washed and then, 50 μl of diluted horseradish peroxidase-conjugated biotin streptavidin complex (Amersham) with HBS at a final dilution of 1:500 was added to each well and allowed to stand for 30 min. After incubation with horseradish peroxidase conjugates, the wells were washed and developed.

2.5. Fluorescence measurements

Fluorescence measurements [13] were performed on a spectrofluorometer (Hitachi) with excitation at 340 nm and emission typically scanned between 350 and 480 nm. Samples were prepared as 1.5 ml total volume in 10 mM HEPES-buffered saline containing 1 mM $CaCl_2$ at pH 7.4. Vesicles were used at 0.42 mM total lipid.

3. Results and discussion

Table 1 summarizes the physicochemical and PC binding properties of the glycosaminoglycans and other acidic polysaccharides employed in this study. Chondroitin sulfate and keratan sulfate, but not heparin and heparan sulfate, were found to bind to PC monolayer. A sensorgram (Biacore profile) of the interaction of chondroitin sulfate with PC immobilized on a sensor chip, which is dose-dependent, is shown in Fig. 1. The addition of other phospholipids, such as PS, PE and PI, to PC decreased the bindings in all cases, indicating that chondroitin sulfate specifically binds to PC and not to other phospholipids. No correlation was found between the sulfate content (Table 1) of chondroitin sulfate and its ability to combine with PC, indicating that binding does not seem to be due to ionic interactions between positive charges of the PC head groups and negative charges of the glycosaminoglycan. The fact that the interaction increases with temperature (Fig. 2) suggests that it is hydrophobic and not ionic. Solid phase binding assays were also performed using ELISA plates. Biotinylated chondroitin sulfate bound to immobilized PC and the binding was inhibited with free unlabeled chondroitin sulfate (Fig. 3) but not with heparin, confirming that the

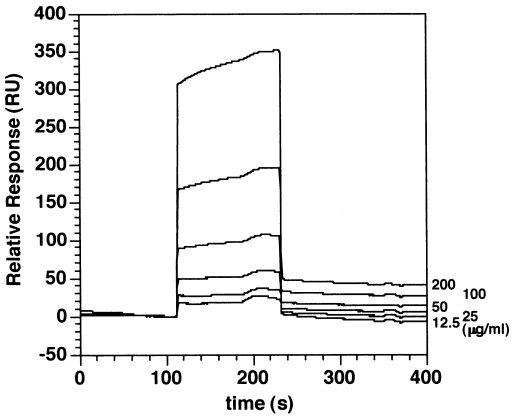

Fig. 1. Sensorgram of chondroitin sulfate binding to immobilized PC. The *y*-axis presents the amount of bound glycosaminoglycan as relative response (resonance units (RU)) and the *x*-axis presented time (in seconds). Various concentrations of chondroitin sulfate solution were flowed over the immobilized PC on HPA sensor chips at a flow rate of 20 μl/min in the presence of 1 mM calcium ion at 25°C.

Table 1
Phosphatidylcholine binding properties of glycosaminoglycans

Glycosaminoglycan	MW[a] ($\times 10^3$)	Sulfate content (%)	Amino sugar	Uronic acid	Sulfation	Bound[b] (RU)
Heparin	13.2	30	GlcN	GlcUA IdoUA	*O*- *N*-	0.0
Heparan sulfate	6.7	9	GlcN	GlcUA IdoUA	*O*- *N*-	0.2
Keratan sulfate	14.9	15	GlcN	Gal	*O*-	27.1
Chondroitin sulfate	8.7	18	GalN	GlcUA	*O*-	5.4
Chondroitin sulfate A	17.2	18	GalN	GlcUA	*O*-	0.0
Chondroitin sulfate C	26.4	18	GalN	GLcUA	*O*-	4.2
Chondroitin	8.9	3	GalN	GlcUA	–	0.0
Dermatan sulfate	18.9	21	GalN	GlcUA IdoUA	*O*-	0.0
Fucoidan	91.2	25		Fuc	*O*-	29.0
Dextran sulfate	69.7	51		Glc	*O*-	0.0
Polygalacturonic acid	N.D.	0		GalUA	–	20.1

GlcN: *N*-acetyl-D-glucosamine; GalN: *N*-acetyl-D-galactosamine; GlcUA: D-glucuronic acid; IdoUA: L-iduronic acid; Gal: D-galactose; Fuc: L-fucose; Glc: D-glucose; GalUA: D-galacturonic acid; RU: relative resonance units; glycosaminoglycan concentration = 0.1 mg/ml, at 25°C; N.D.: not determined.

[a]Molecular weights were established by DAWN analysis.

[b]Bound was obtained by Biacore analysis.

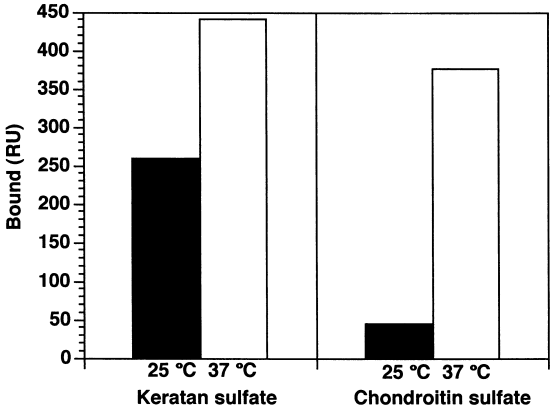

Fig. 2. Effect of temperature on binding of glycosaminoglycan to PC. The *y*-axis presents relative amounts of binding (Bound, in RU). 1 mg/ml of chondroitin sulfate or keratan sulfate solution was flowed over the immobilized PC on HPA sensor chips at a flow rate of 20 μl/min in the presence of 1 mM calcium ion at 25°C or 37°C.

interaction is specific. Keratan sulfate and chondroitin sulfate interactions with PC have a similar ΔG (Table 2).

The aggregation of PC by chondroitin sulfate was confirmed by measurement of lipid dynamics in vesicles containing phosphatidylglycerol, pyrene-labelled phosphatidylglycerol and PC. Changes in the lipid dynamics of the vesicles were successfully monitored by measuring the red shift of the fluorescence induced upon formation of pyrene dimers (excimer). The fluorescence of pyrene-phosphatidylglycerol monomer was integrated between 394 and 420 nm, while excimer emission was integrated between 474 and 482 or 470 and 490 nm. When chondroitin sulfate was allowed to react with phosphatidylglycerol/PC lipid membranes, the level of pyrene dimer was dramatically increased as shown in Fig. 4. The results indicate that chondroitin sulfate segregates the PC molecules, restricts their lateral mobility and forms the phosphatidylglycerol-rich domain where the formation of pyrene dimers is induced.

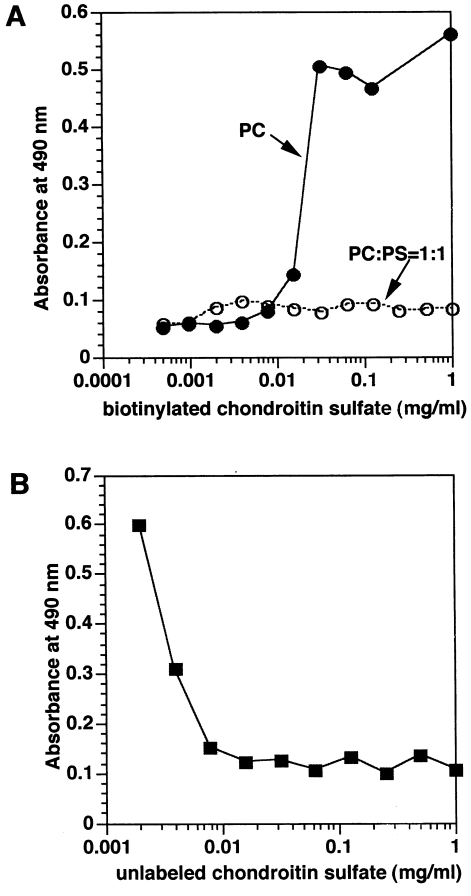

Fig. 3. Interaction between chondroitin sulfate and PC and its inhibition assayed by ELISA. The *y*-axis presents relative amount of bound chondroitin sulfate as absorbance at 490 nm by color development of horseradish peroxidase. Chondroitin sulfate bound to immobilized PC on ELISA plate in a dose-dependent manner (A). The binding of chondroitin sulfate to PC was inhibited by the addition of unlabeled chondroitin sulfate (B).

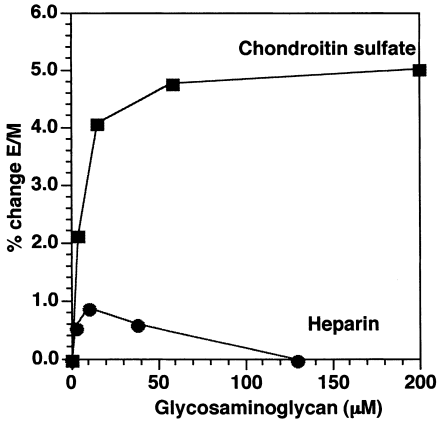

Fig. 4. Measurement of lipid dynamics in vesicles by pyrene fluorescence. Changes in the dynamics of lipid molecule phosphatidylglycerol/PC vesicles were detected using the fluorescence of a phosphatidylglycerol derivative containing pyrene in one acyl chain (pyrene-phosphatidylglycerol, Molecular Probes). The measurement of monomer (M) and excimer (E) emissions is proportional to the respective total emissions; also the calculated percent changes in E/M, [(E/M)final−(E/M)initial]/(E/M)initial×100%, are accurate since the proportionalities cancel out. The percent change in E/M was sufficient for analyzing the changes in pyrene-PC dynamics to measure lateral diffusion of phospholipids in vesicles [16]. The figure shows percent change in monomer and excimer fluorescence intensities between scans of pyrene-phosphatidylglycerol/PC vesicles. Data reflect the average of three determinations.

In the course of our study, a sulfated fucose polymer, fucoidan, which is not a member of mammalian glycosaminoglycans, was also found to bind to PC. This polysaccharide is of interest since it was found to possess antiproliferative and antitumor properties, to inhibit tumor metastasis [14–16], and to induce apoptosis in HL-60 cells (our unpublished results). These properties have been ascribed to the interference by fucoidan of the passage of the tumor cells through the capillary wall by the sulfate groups on the polysaccharides. Our results lead us to propose that the formation of PC microdomains by glycosaminoglycan binding to OC may be involved in this phenomenon. The exposure of other microdomains such as PS microdomains induced by the formation of PC microdomains on the tumor cells may cause apoptosis.

Our model proposed in Fig. 5 implies that chondroitin sulfate and keratan sulfate are like vines on the ground rather than the crown of a tall tree and contribute to the formation of PC microdomains in the outer leaflet of the cell membrane. Hakomori and coworkers have reported that glycosphingolipids consisting of ceramide and carbohydrate residues form

Table 2
Kinetic constants of the interaction of glycosaminoglycans with PC at 37°C

Glycosaminoglycan	K_d (nM)	k_d (s^{-1})	k_a (M^{-1} s^{-1})	ΔG (kJ mol^{-1})[a]
Keratan sulfate	6.3×10^{-8}	2.0×10^{-4}	3.2×10^{3}	−43
Chondroitin sulfate	1.0×10^{-7}	1.5×10^{-4}	1.5×10^{3}	−41

[a]$\ln(1/K_d) = -\Delta G/RT$ ($R = 8.31$ JK mol^{-1}, $T = 298$ K or 310 K).

microdomains as structural and functional units and some of the signal transductions are assumed to be directly induced through microdomains of glycosphingolipids (reviewed by Hakomori et al. [17]). In this case, glycosphingolipids seem to associate laterally with one another through weak interactions among the carbohydrate heads of the lipids. With a different approach, Jacobson et al. found that glycosphingolipids preferentially associate with glycosyl-phosphatidyl-inositol-anchored proteins, such as Thy-1 [18,19]. Although glycosaminoglycans of membrane proteoglycan have been assumed to cover most of the outer surface of the cell including the membrane glycolipids, the chondroitin sulfate/keratan sulfate–PC microdomains proposed in this study may not only contribute to strengthening the membrane structure but also to the efficient exposure on the cell surface of glycolipid microdomains or other domains such as PS-rich ones.

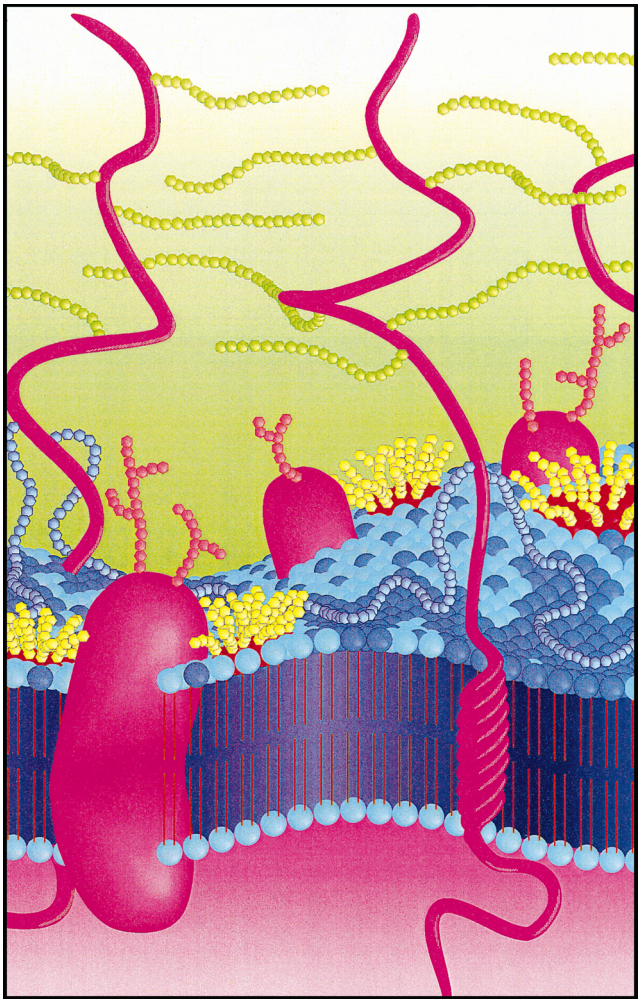

Fig. 5. Proposed model of organization of PC and glycosaminoglycan in the plasma membrane. Clusters of PC (dark blue) and glycosphingolipids (red, with yellow oligosaccharides) are present on the outer leaflet of the plasma membrane. Other membrane lipids are light blue. Protein cores of glycoproteins and proteoglycans and *N*-linked oligosaccharides on glycoproteins are magenta. Chondroitin sulfate and keratan sulfate chains are violet. Heparan sulfate chains are green.

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