Biochimica et Biophysica Acta, 599 (1980) 301—314

BBA 78751

CELL SURFACE PROTEOGLYCANS AS A NEGATIVE MODULATOR IN CONCANAVALIN A-MEDIATED AGGLUTINATION OF HEPATOMA CELLS

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(Received November 20th, 1979)

Key words: Agglutination; Concanavalin A; Cell surface; Proteoglycan; (Hepatoma)

Summary

Proteoglycan (heparan sulfate-protein conjugate) was solubilized with 8 M urea from rat liver plasma membranes after enzymic (RNAase, neuraminidase) treatments and extensively purified by chromatography and gel filtration. The final products gave an average ratio of hexuronate to protein (weight) of approx. 1.5, contained hexosamine equimolar to hexuronate and were sensitive to β-elimination (the molecular weight being reduced from $20 \cdot 10^4$ to $3 \cdot 10^4$ (gel filtration)).

The proteoglycan fraction, when added to trypsinized and untrypsinized ascites hepatoma (AH-130F(N)) cells, inhibited the concanavalin A-mediated agglutination of the cells. However, the alkali-treated proteoglycan (β-elimination) or acid mucopolysaccharide fraction prepared from liver plasma membranes by papain digestion were less effective, and a reference preparation of heparan sulfate was almost ineffective. It was confirmed that significant amounts of proteoglycan labelled with $^{35}SO_4^{2-}$ were firmly bound to or taken up by the trypsinized ascites hepatoma cells.

These results together with the sensitization of lectin-mediated agglutination by mild protease treatment of cells suggest that cell surface proteoglycans may act as a negative modulator in the lectin-mediated agglutination of cells.

Introduction

The malignant (or transformed) cells are usually more sensitive to lectin-mediated agglutination than the normal cells [1—3]. Although a number of hypotheses have been advanced to explain the difference between the two

types of cells in the sensitivity to lectin-mediated agglutination [4,5], no general mechanism applicable to a variety of cells has emerged. On the other hand, normal cells (or even transformed cells as well) are known to become sensitive to the lectin-mediated agglutination by mild protease treatment [2,3,6,7] that modifies the cell surface architecture, liberating acid mucopolysaccharide(s) [8] and/or some glycopeptides from the cell surface. We have reported earlier that the acid mucopolysaccharides liberated from liver cell surfaces are mainly heparan sulfate, whereas those from ascites hepatoma cells are rich in chondroitin sulfate and others, which seem to have smaller affinities for Ca^{2+} as compared to heparan sulfate [9].

In earlier papers [10,11] we have suggested the possibility that cell surface proteoglycnas may behave as a negative modulator in the lectin-mediated cell agglutination. In order to substantiate this speculation, proteoglycan was prepared from rat liver plasma membranes and tested for the effect on concanavalin A-mediated agglutination of trypsinized or untrypsinized ascites hepatoma cells in the present study.

Materials and Methods

Isolation of proteoglycan from plasma membranes of rat livers. Plasma membranes were prepared from livers (approx. 260 g) of male Donryu rats (body weight 140—180 g) fasted overnight according to the method of Ray [12]. The plasma membranes were suspended in 30 ml of 0.1 M potassium acetate buffer (pH 5.5) containing 1 mM $CaCl_2$, 30 μg/ml leupeptin (antiprotease) [13] and 100 units of neuraminidase, and the suspension was incubated at 37°C for 3 h, followed by centrifugation. The pellet was suspended in 20 ml of 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM EDTA, 30 μg/ml leupeptin and 100 units of RNAase T_1, and the suspension was incubated at 37°C for 3 h. After centrifugation, the pellet was resuspended in 30 ml of 8 M urea containing 50 mM $NaHCO_3$, 1 mM mercaptoethanol and 1 mM EDTA (pH 10.0) and the suspension was incubated at 4°C for 3 h with gentle stirring, followed by centrifugation at 30 000 rev./min for 90 min (Spinco 30 rotor). The extraction procedure was repeated once more. The combined extracts were dialyzed against 0.5 M urea/10 mM phosphate buffer (pH 8.0).

The dialyzate was concentrated by ultrafiltration using a G05-T diaphragm that passes molecules of less than 5000 molecular weight, and then applied to a column (1.6 × 21 cm) of DEAE-cellulose (DE52) equilibrated with 0.5 M urea/10 mM phosphate buffer (pH 8.0). The column was thoroughly washed with the 0.5 M urea/phosphate buffer containing 0.25 M NaCl, and then washed with a linear NaCl gradient (0.25—2.0 M) in the same urea/phosphate solution. Fractions were collected every 5 ml effluent, monitoring A_{280} nm, A_{260} nm, and hexuronic acid.

The fractions containing hexuronic acid (with a peak at 0.4 M NaCl) were combined, concentrated by ultrafiltration and then subjected to gel filtration through a column (1.1 × 90 cm) of Sephadex G-100 prewashed with 0.5 M urea/0.5 M NaCl/10 mM phosphate buffer (pH 7.2), using the same urea/NaCl/phosphate buffer. Fractions containing hexuronic acid (pass-through fractions) were combined, condensed by ultrafiltration and then subjected to gel filtra-

tion through a column (1.6 × 78 cm) of Sepharose 6B. Hexuronic acid-containing fractions were combined and dialyzed against deionized water (sample A).

Sample A thus prepared was subjected to affinity chromatography using concanavalin A-Sepharose 4B so that concanavalin A-binding glycoproteins, if there were any, are eliminated. Concanavalin A-Sepharose 4B, which had been thoroughly washed with 1 M NaCl/10 mM phosphate buffer (pH 7.2), was layered upon an equal size (1.1 × 6 cm) of Sephadex G-50 to trap any concanavalin A molecules which happened to leak from the concanavalin A-Sepharose column. An aliquot of sample A was applied to the column of double-layered concanavalin A-Sepharose and Sephadex G-50, followed by elution with the 1 M NaCl/10 mM phosphate buffer (pH 7.2). The hexuronic acid-containing material was recovered near the void volume (sample B).

Preparation of $^{35}SO_4^{2-}$-labelled proteoglycan fraction. Each of two rats received two intraperitoneal injections of $^{35}SO_4^{2-}$ (total 1.5 mCi) with a 10 h interval, and the rats were killed 14 h after the last injection. $^{35}SO_4^{2-}$-labelled plasma membranes (equivalent to 28 mg protein) were prepared from the livers of these rats and they were mixed with unlabelled liver plasma membranes (equivalent to 128 mg protein). The $^{35}SO_4^{2-}$-labelled proteoglycan fraction was prepared from these plasma membranes by a slightly simplified method, in which the gel filtration procedure in preparing sample A was omitted.

Preparation of acid mucopolysaccharide (protein-free) from plasma membranes. Plasma membranes prepared from 275 g of livers were suspended in 100 ml of 0.2 M sucrose/20 mM mercaptoethanol/1 mM EDTA in 50 mM phosphate buffer (pH 6.8), and the suspension was incubated at 37° C for 10 min in the presence of 3 units of papain [9] and then centrifuged (27 000 rev./min for 1 h). The supernatant was dialyzed against 10 mM phosphate buffer (pH 8.0) and the dialyzed solution was concentrated by ultrafiltration and finally applied to a column (0.7 × 29 cm) of DEAE-cellulose equilibrated with the same buffer. The column was successively washed with the phosphate buffer, 0.2 M NaCl in the same buffer and then a linear NaCl gradient (0.2—2.0 M) in the same buffer. Hexuronate-containing fractions with a peak at 0.4 M NaCl were combined and dialyzed against deionized water.

Alkali treatment of proteoglycan (β-elimination). An aliquot of sample A was treated with 0.5 N NaOH at 4° C for 24 h, and then the reaction mixture was neutralized with hydrochloric acid, followed by dialysis against deionized water. The dialyzed solution was subjected to gel filtration through Sepharose 6B as described above to check the β-elimination.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteoglycans or acid mucopolysaccharides. 50 μl of sample solution in the 10 mM phosphate buffer, pH 8.5, were mixed with an equal volume of fluorescamine solution (0.5 mg fluorescamine/ml Me_2SO) to stain proteins according to the method of Ragland et al. [14] with a slight modification. 10 μl of 10% sodium dodecyl sulfate solution containing 1% mercaptoethanol were added to a sample solution and the mixture was heated at 100° C for 3 min. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed on a 7.5% cylindrical gel according to the method of Weber and Osborn [15]. Fluorescamine-bound protein bands were observed under an ultraviolet lamp. The gels were then removed from tubes, immersed in 5% methanol/7.5% acetic acid

overnight to remove sodium dodecyl sulfate and then acid mucopolysaccharide bands were stained with 0.1% Alcian blue in 1% acetic acid.

Trypsinization of ascites hepatoma cells. Ascites hepatoma AH-130F(N) cells were harvested from peritoneal cavities of male Donryu-strain rats (6—7-weeks old) 5 days after inoculation of the cells and were freed from blood cells by repeated washing with the phosphate-buffered saline. The hepatoma cells were then suspended ($1—2 \cdot 10^7$ cells/ml) in the phosphate-buffered saline containing 10 μg/ml trypsin and the suspension was incubated at 37°C for 2 min. The trypsinized cells were spun down and washed with the phosphate-buffered saline.

Agglutination tests. When the effect of proteoglycan or acid mucopolysaccharide on concanavalin A-mediated agglutination of cells was tested, a suspension of trypsinized AH-130F(N) cells ($1.5 \cdot 10^6$ cells/ml) in 90 μl of the phosphate-buffered saline was preincubated with or without a test material in a small test-tube at 25°C for 30 min with gentle shaking, and then 10 μl of concanavalin A solution in the phosphate-buffered saline (20 μg concavalin A/ml) were added to the cell suspension in each tube. The mixture was incubated at 25°C. At a scheduled interval of incubation an aliquot of the suspension was placed in a hematocytometer to count the number of free cells. Cells in less than four-cell clusters were counted as free cells in accordance with the procedures described in the earlier paper [10]. Only in the case of untrypsinized cells, test-tubes were previously coated with bovine serum albumin to avoid the cellular adhesion to glass: (coating test-tubes with albumin had been confirmed as not altering the concanavalin A-mediated agglutination of trypsinized cells).

Binding of $^{35}SO_4^{2-}$-labelled proteoglycan to trypsinized cells. Trypsinized AH-130F(N) cells were suspended in 110 μl of the phosphate-buffered saline ($2 \cdot 10^7$ cells/ml) containing the $^{35}SO_4^{2-}$-labelled proteoglycan fraction (3—8 μg hexuronic acid equivalent/ml) and the whole mixture was incubated at 25°C for 90 min. After incubation the suspension was added to 4 ml of the phosphate-buffered saline, followed by centrifugation at $500 \times g$ for 20 s. The cellular pellet was twice washed with 4 ml of the phosphate-buffered saline and finally counted for radioactivity.

Assays and reagents. Protein was assayed by the method of Lowry et al. [16] with bovine serum albumin as standard. Hexuronic acid was assayed by the method of Galambos [17] with D-glucuronic acid as standard. Hexosamine was assayed by the method of Svennerholm [18] with D-glucosamine hydrochloride as standard. Sialic acids were assayed by the periodate-resorcinol method of Jourdian et al. [19] with *N*-acetylneuraminic acid as standard. Neutral sugar was assayed by the method of Morris [20] with a slight modification (i.e., a mixture of 2.5 ml of 0.2% anthrone in 13.5 M H_2SO_4 and 0.25 ml of a sample solution were heated at 100°C for 10 min, followed by spectrophotometry at 620 nm) with D-glucose as standard. RNA was assayed by the method of Fleck and Munro [21]. Radioactivity due to $^{35}SO_4^{2-}$ incorporated into proteoglycan or cells was counted in a liquid scintillation spectrometer (ALOKA, LSC-651) using 2,5-diphenyloxazole/toluene/Cellosolve as a scintillation fluid after the samples were oxidized with perchloric acid and H_2O_2.

RNAase T_1 (8000 units/mg protein) was a gift from Sankyo Pharm. Co. Ltd., Tokyo. Trypsin (type I, twice crystallized), papain (twice crystallized), con-

canavalin A (grade III) and α-methyl-D-mannoside (grade II) were purchased from Sigma Chemical Co., St. Louis, MO. Neuraminidase from *Vibrio cholerae* 500 units/ml) was from BDH, Poole, U.K. Heparin (167 units/mg) and heparan sulfate were from Wako Pure Chem. Ind. Ltd., Osaka, Japan and from Upjohn Co., Kalamazoo, U.S.A., respectively. Leupeptin was a gift from the Institute for Microbial Chemistry, Tokyo, Japan. Fluorescamine was from Nippon Roche K.K., Tokyo, Japan. $Na_2{}^{35}SO_4$ (121.4 Ci/mol) was from Radiochemical Centre, Amersham, U.K. DEAE-cellulose DE52 was from Whatman Ltd., Maidstone, U.K. Sephadex G-100, Sepharose 6B, fluoresceinisothiocyanate-dextrans and concanavalin A-Sepharose 4B were from Pharmacia Fine Chem., Uppsala, Sweden.

Results

Isolation and purification of proteoglycan from plasma membranes

The proteoglycan preparations (samples A and B) were prepared from plasma membranes of rat livers as described in Materials and Methods. In the preliminary experiments we have confirmed that more than 80% of RNA originally associated with plasma membranes [22] is eliminated by RNAase T_1 digestion and approx. 70% of sialic acids in plasma membranes is eliminated by neuraminidase digestion. Such enzymic treatments of plasma membranes prior to extraction of proteoglycan are useful for minimizing the contamination of RNA and sialoglycoproteins into proteoglycan extracts.

Although the exact estimation of hexuronic acid (a common component in most acid mucopolysaccharides) in plasma membranes is rather difficult mainly because of the interference with lipid constituents, it seems that approx. 30—40% of hexuronic acid associated with liver plasma membranes are recovered in the extract by the urea/bicarbonate/mercaptoethanol/EDTA solution as calculated from the hexuronic acid content of defatted plasma membranes. The weight ratio of hexuronic acid to protein in the extract was approx. 1 : 1000.

When the urea extract was subjected to chromatography on DEAE-cellulose, most of the proteins and neutral sugars were eluted before the hexuronic acid-containing fraction, which was eventually eluted with a peak at 0.4 M NaCl as shown in Fig. 1. At this stage of purification, the hexuronic acid to protein ratio was approx. 1 : 5. When the hexuronic acid-containing fraction was next subjected to gel filtration through Sephadex G-100, a hexuronic acid-containing band was eluted near the void volume, apparently being freed from most of RNA fragments as shown in Fig. 2. The ratio of hexuronic acid to protein was then 1.5 : 1.

The void volume fraction containing hexuronic acid was next subjected to gel filtration through Sepharose 6B. As shown in Fig. 3 (Fig. 3a), a hexuronic acid-containing fraction (sample A) was eluted with a peak at the position corresponding to a molecular weight of $20 \cdot 10^4$. The ratio of hexuronic acid to protein in sample A remained unaltered as compared to the ratio at the preceding step. The recoveries of hexuronic acid and other components in the course of purification are summarized in Table I.

Characterization of proteoglycan prepared from rat liver plasma membranes

As described above, the proteoglycan preparation (sample A) gave an average

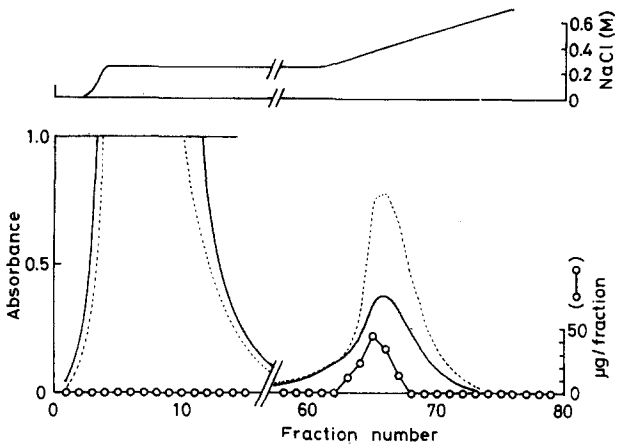

Fig. 1. Chromatography of the urea extract from liver plasma membranes on DEAE-cellulose. Plasma membranes treated with RNAase T_1 and neuraminidase were extracted with urea/bicarbonate/mercaptoethanol/EDTA. The extract was dialyzed against urea/NaCl in phosphate buffer, and the dialyzed solution was then subjected to chromatography on DEAE-cellulose as described in Materials and Methods. After application of the sample, the column was successively washed with 0.5 M urea/0.25 M NaCl/10 mM phosphate buffer (pH 8.0) and then with a linear NaCl gradient (0.25—2.0 M) in the same urea/phosphate buffer solution. Fractions were collected every 5 ml effluent, monitoring $A_{280\ nm}$ (———), $A_{260\ nm}$ (· · · · · ·) and hexuronic acid (μg/fraction) (o———o).

ratio of hexuronic acid to protein of approx. 1.5, and equimolar hexuronic acid and hexosamine (Table I) in contrast to negligibly small amounts of other sugars. These results suggest an approximate ratio (weight) of acid mucopolysaccharide (heparan sulfate) moiety to protein moiety of approx. 3—4 : 1. Sample A appears to have an approximate molecular weight (average) of approx. $20 \cdot 10^4$ (Fig. 3a) although the elution profile in gel filtration through Sepharose 6B was rather broad, suggesting a heterogeneity with respect to molecular weight (Fig. 3a). As shown in Fig. 3b, sample A was sensitive to alkali (β-elimination), reducing the molecular weight to $3 \cdot 10^4$. The results sug-

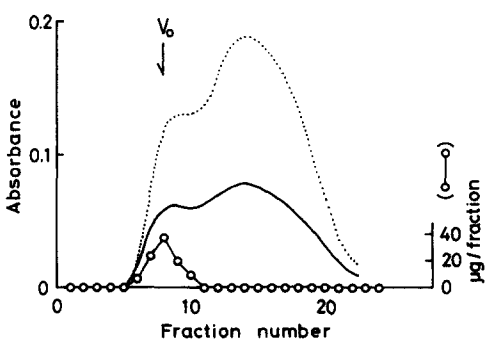

Fig. 2. Gel filtration of the proteoglycan fraction in Fig. 1 through Sephadex G-100. The fractions containing hexuronic acid in Fig. 1 (fraction Nos. 63—67) were combined, concentrated by ultrafiltration, and then subjected to gel filtration as described in Materials and Methods. Elution was performed by running 0.5 M urea/0.5 M NaCl/10 mM phosphate buffer (pH 7.2), and fractions were collected every 5 ml effluent, monitoring $A_{280\ nm}$ (———), $A_{260\ nm}$ (· · · · · ·) and hexuronic acid (μg/fraction) (o———o).

TABLE I

COMPOSITIONS OF PROTEOGLYCAN FRACTIONS AT VARIOUS PURIFICATION STEPS

The general procedures for extraction and purification of proteoglycan(s) from liver plasma membranes are described in Materials and Methods. n.d. = not determined; NANA, *N*-acetylneuraminic acid.

Step	Protein (μg)	Hexuronate (μg)	Hexosamine (μg)	Hexose (μg)	RNA (μg)	NANA (μg)
1 Plasma membranes (starting material)	$550 \cdot 10^3$	450	n.d.	$4.1 \cdot 10^3$	$8.5 \cdot 10^3$	$2.7 \cdot 10^3$
2 Urea extract	$140 \cdot 10^3$	160	n.d.	230	n.d.	170
3 DEAE-cellulose (chromatography)	688	127	n.d.	n.d.	n.d.	trace
4 Sephadex G-100 (gel filtration)	66	102	n.d.	n.d.	n.d.	n.d.
5 Sepharose 6B (gel filtration) (sample A)	57	84	91	trace	trace	n.d.

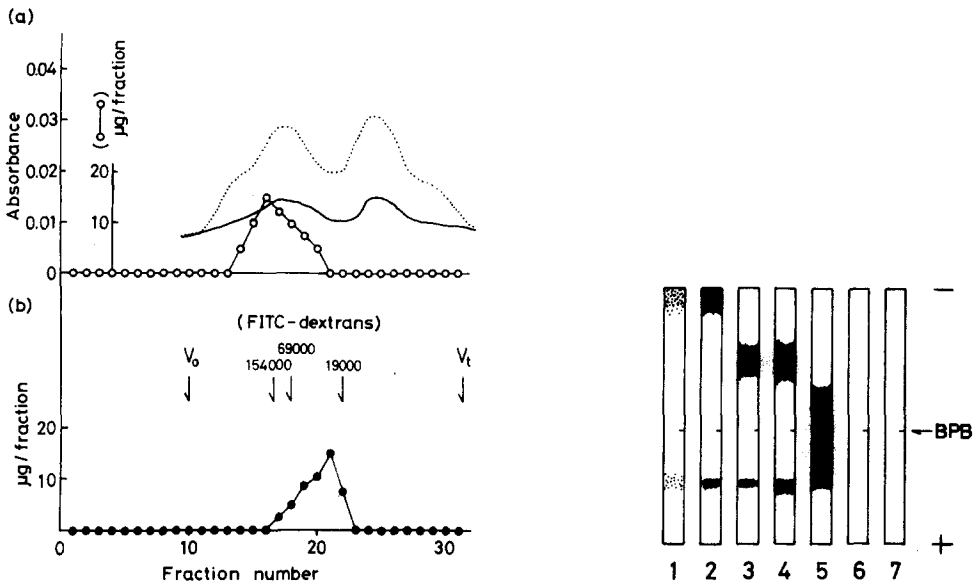

Fig. 3. Gel filtration of the proteoglycan fraction in Fig. 2 and the alkali-treated sample A through Sepharose 6B. (a) The hexuronic acid-containing fractions in Fig. 2 were combined, concentrated by ultrafiltration and then subjected to gel filtration through Sepharose 6B as described in the text, monitoring $A_{280\ nm}$ (———), $A_{260\ nm}$ (· · · · ·) and hexuronic acid content in each fraction. The hexuronic acid-containing band thus separated was referred to as sample A (o———o). (b) In order to examine the O-glycosidic linkage between protein and glycan moieties, sample A was treated with 0.5 N NaOH at 4°C for 24 h (β-elimination), neutralized and then dialyzed. An aliquot of the dialyzed solution was subjected to gel filtration through Sepharose 6B under the same conditions, monitoring hexuronic acid in each fraction (•———•). Arrows indicate positions of reference preparations of fluoresceinisothiocyanate (FITC)-dextrans with known molecular weights.

Fig. 4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of sample A, alkali-treated sample A, heparan sulfate and heparin. Samples labelled with fluorescamine were subjected to sodium dodecyl sulfate-polyacrylamide (7.5%) gel electrophoresis as described in Materials and Methods under the conditions of room temperature, 2 h and 6 mA/gel. After electrophoresis, the gels were observed either under an ultraviolet lamp or after staining with Alcian blue. The migration was from top to bottom in each gel (No. 1—7). As controls (Nos. 6 and 7) the media without samples were treated similarly and subjected to electrophoresis. (1) Sample A (equivalent to 3.5 μg protein or to 5.2 μg hexuronic acid), under an ultraviolet lamp). (2) Sample A (equivalent to 1.7 μg hexuronic acid), after Alcian blue staining. (3) Alkali-treated sample A (equivalent to 1.4 μg hexuronic acid), after Alcian blue staining. (4) Heparan sulfate (equivalent to 1.5 μg hexuronic acid), after Alcian blue staining. (5) Heparin (equivalent to 1.5 μg hexuronic acid), after Alcian blue staining. (6) Blank control, under an ultraviolet lamp. (7) Blank control, after Alcian blue staining. BPB, bromophenol blue used as a marker.

gest that the proteoglycan may contain several acid mucopolysaccharide chains (each with approx. $3 \cdot 10^4$ molecular weight) attached to a protein moiety (with approx. $4—5 \cdot 10^4$ molecular weight) through O-glycosidic linkages.

The alkali sensitivity of proteoglycan was also confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. As shown in Fig. 4, sample A pretreated with fluorescamine have a major fluorescent band near the gel top in addition to a minor band with a much greater mobility. These bands were also stained with Alcian blue, suggesting that they are acidic glycan-protein conjugates. When the alkali-treated sample A was subjected to electrophoresis under the same conditions, a major Alcian blue-stainable band was found to migrate

through the gel with an electrophoretic mobility similar to that of a reference preparation of heparan sulfate (but differing from that of heparin). A minor band with a greater mobility was commonly detected in the alkali-treated sample A and reference preparations of heparan sulfate (and also heparin) similarly to untreated sample A. The identity of the major acid mucopolysaccharide component in sample A as heparan sulfate was also confirmed by electrophoresis on a cellulose acetate strip (data not shown; but refer to Ref. 23) although the identity of the minor component remains to be elucidated.

Effects of proteoglycans and acid mucopolysaccharides on concanavalin A-mediated agglutination of trypsinized and untrypsinized AH-130F(N) cells

In the preliminary experiments we have found that the sensitivity of AH-130F(N) cells to the concanavalin A-mediated agglutination is markedly enhanced after the mild trypsin treatment of the cells (10 μg trypsin/ml, 37°C, 2 min). Untrypsinized AH-130F(N) cells showed only a 40% reduction of free cells after 60 min of incubation with concanavalin A at 2 μg/ml, whereas trypsinized AH-130F(N) cells were almost completely agglutinated under the same conditions. Even in the presence of concanavalin A at 20 μg/ml, untrypsinized cells did not show a reduction of larger than 60% of free cells.

The mild protease treatment is known to digest the cell coat (glycocalyx) structure, liberating acid mucopolysaccharides from the cell surface [8,9]. To examine the effect of externally added proteoglycan on the lectin-induced cell agglutination, the cells freed from cell coats ('naked cells') thus appeared to be more convenient than the intact cells which have their own cell coats. Therefore, in the present study most of the experiments were carried out with trypsinized AH-130F(N) cells. AH-130F(N) cells (trypsinized or untrypsinized) were preincubated with test materials (proteoglycan or acid mucopolysaccharides) at 25°C for 30 min, and then concanavalin A was added at a concentration of 2 μg/ml (trypsinized cells) or 20 μg/ml (untrypsinized cells), and the kinetics of cell agglutination occurring thereafter were observed.

Fig. 5a shows the kinetics of agglutination of trypsinized cells preincubated with sample A or alkali-treated sample A at various concentrations, indicating the dose-dependent inhibition of concanavalin A-mediated cell agglutination by sample A. On the other hand, the alkali-treated sample A even at a concentration of 27 μg hexuronic acid equivalent/ml showed a much weaker effect as compared to the intact sample A at the same hexuronic acid concentration, suggesting the requirement of a protein moiety for the complete activity. Sample B, which had been passed through a concanavalin A-Sepharose column, also showed a dose-dependent inhibition of concanavalin A-mediated agglutination of trypsinized cells similarly to sample A as shown in Fig. 5b. The proteoglycan inhibition of concanavalin A-mediated agglutination was also observed with the intact AH-130F(N) cells, although the agglutination was less marked as compared to the trypsinized cells even in the presence of concanavalin A at a much higher concentration as shown in Fig. 5c.

As summarized in Table II, acid mucopolysaccharide released from liver plasma membranes by papain treatment as well as the alkali-treated sample A showed only a slight inhibitory activity. The reference preparation of heparan sulfate did not affect concanavalin A-mediated agglutination even at a concen-

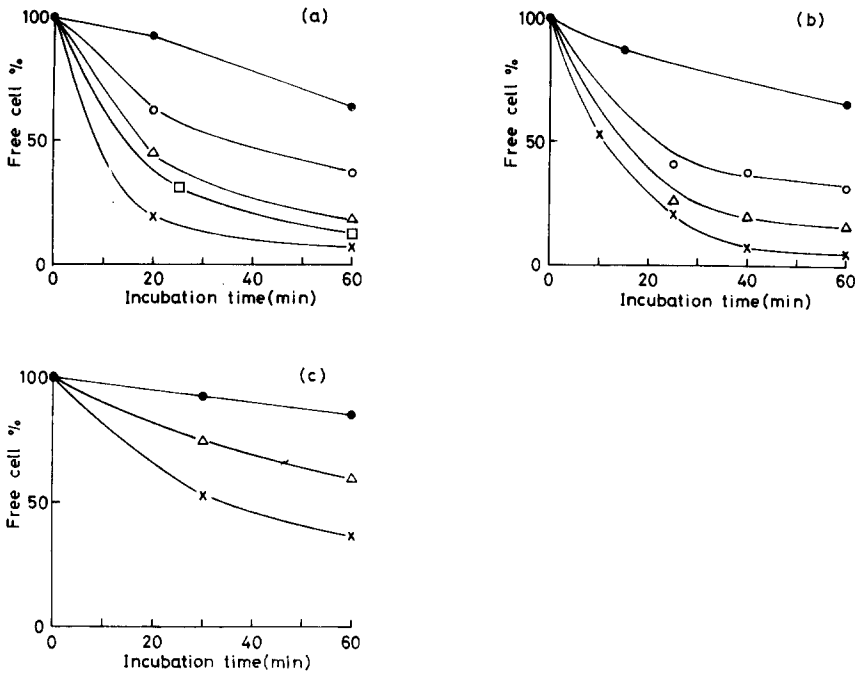

Fig. 5. Kinetics of concanavalin A-mediated agglutination of AH-130F(N) cells (trypsinized or untrypsinized) preincubated with proteoglycan preparations. The general procedures for preincubation of cells with a proteoglycan preparation and following concanavalin A-mediated agglutination of cells are described in Materials and Methods. Ordinate: percent free cells; abscissa: time after addition of concanavalin A. (a) Trypsinized AH-130F(N) cells were preincubated for 30 min with sample A at 27 μg hexuronate/ml (○———○), 18 μg hexuronate/ml (△———△) and 0 μg hexuronate/ml (X———X), or with alkali-treated sample A at 27 μg hexuronate/ml (□———□) and then concanavalin A was added at 2 μg/ml. ●———●, spontaneous agglutination (in the absence of concanavalin A) of cells preincubated with the phosphate-buffered saline only (without proteoglycan). (b) Trypsinized AH-130F(N) cells were preincubated with sample B at 32 μg hexuronate/ml (○———○), 21 μg hexuronate/ml (△———△) and 0 μg hexuronate/ml (X———X) and then concanavalin A was added at 2 μg/ml. ●———●, spontaneous agglutination (in the absence of concanavalin A) of cells preincubated with the phosphate-buffered saline only. (c) Intact AH-130F(N) cells were preincubated with sample A at 13 μg hexuronate/ml (△———△) and 0 μg hexuronate/ml (X———X), and then concanavalin A was added at 20 μg/ml. ●———●, the spontaneous agglutination. One point is an average of duplicate countings.

tration as high as 120 μg hexuronic acid equivalent/ml. In this table, it is also shown that the concanavalin A-mediated agglutination of cells is completely prevented by α-methyl-D-mannoside at 100 mM.

In order to examine whether the inhibitory effect of proteoglycan may be reversible or not, trypsinized AH-130F(N) cells were preincubated with sample A (at 13 and 26 μg hexuronic acid equivalents/ml) in 60 μl of the phosphate-buffered saline at 25°C for 30 min, and then spun down gently. After pipetting off 40 μl of the supernatant, the rest was mixed with 160 μl of the phosphate-buffered saline so that the concentration of sample A in the resulting cell suspension fell far below the effective concentrations. Concanavalin A in 20 μl of the phosphate-buffered saline was then added so that the final concentrations of concanavalin A and cells became 2 μg/ml and $1.5 \cdot 10^6$ cells/ml, respectively. The cell agglutination kinetics shown in Fig. 6 indicate that the cells pre-

TABLE II

EFFECTS OF ACID MUCOPOLYSACCHARIDES PREPARED FROM PLASMA MEMBRANES BY PAPAIN TREATMENT, HEPARAN SULFATE, AND α-METHYL-D-MANNOSIDE ON CONCANAVALIN A-MEDIATED AGGLUTINATION OF TRYPSINIZED AH-130F(N) CELLS

Trypsinized AH-130F(N) cells were preincubated with the samples at various concentrations as indicated at 25°C for 30 min prior to addition of 2 μg/ml concanavalin A. Free cells were counted at 0 and 60 min of incubation at 25°C after addition of concanavalin A. In this table, percent free cells are expressed as ratio of free cells at the end of incubation to those at 0 time of incubation. Average of duplicate countings.

Expt. No.		Concentration of sample		Concanavalin A (μg/ml)	% free cells
		μg hexuronate/ml	mM		
I	Papain-AMPS *	0	—	0	81
		0	—	2	12
		17	—	2	22
		26	—	2	24
II	Heparan sulfate	0	—	0	87
		0	—	2	20
		6	—	2	18
		60	—	2	22
		120	—	2	20
	α-Methyl-mannoside	—	0.1	2	15
		—	1.0	2	18
		—	10	2	59
		—	100	2	83

* Acid mucopolysaccharide prepared from liver plasma membranes by papain digestion as described in the text.

incubated with sample A retain the resistance to concanavalin A-mediated agglutination even after a large dilution of sample A.

Binding of $^{35}SO_4^{2-}$-labelled proteoglycan to trypsinized AH-130F(N) cells

In order to investigate whether the externally added proteoglycan prepared

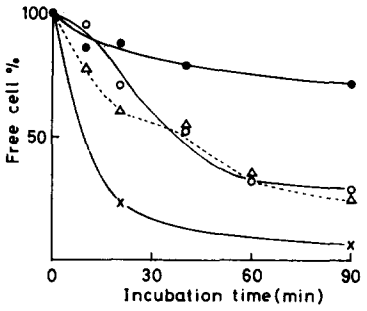

Fig. 6. Kinetics of concanavalin A-mediated agglutination of trypsinized AH-130F(N) cells preincubated with proteoglycan when an excess of proteoglycan was removed prior to the addition of concanavalin A. Trypsinized AH-130F(N) cells were preincubated with sample A at 26 μg hexuronic acid/ml (○———○), 13 μg hexuronate acid/ml (△-------△) at 25°C for 30 min, followed by centrifugation. Most of the supernatant was removed by pipetting. The cells were resuspended in the phosphate-buffered saline and concanavalin A was added at 2 μg/ml. X———X, concanavalin A-mediated agglutination of cells preincubated without proteoglycan, and ●———●, spontaneous cell agglutination (in the absence of concanavalin A). One point is an average of duplicate countings.

TABLE III

BINDING OF $^{35}SO_4^{2-}$-LABELLED PROTEOGLYCAN TO TRYPSINIZED AH-130F(N) CELLS

A mixture of $^{35}SO_4^{2-}$-labelled proteoglycan (2200 cpm/μg hexuronic acid) at various concentrations, as indicated, and trypsinized AH-130F(N) cells at $2 \cdot 10^7$ cells/ml in 110 μl of phosphate-buffered saline was incubated at 25°C for 90 min. After incubation, 4 ml of the phosphate-buffered saline were added to the mixture and the cells were spun down, twice washed with 4 ml phosphate-buffered saline and counted for ^{35}S radioactivity.

Expt. No.	$^{35}SO_4^{2-}$-labelled proteoglycan added		$^{35}SO_4^{2-}$-labelled proteoglycan bound	
	cpm/ml	μg hexuronate/ml	cpm/10^7 cells	μg hexuronate/10^9 cells
I	$176 \cdot 10^2$	8	73	3.3
	$88 \cdot 10^2$	4	63	2.8
II	$132 \cdot 10^2$	6	59	2.7
	$66 \cdot 10^2$	3	36	1.6

from liver plasma membranes may actually be taken up by trypsinized AH-130F(N) cells, the $^{35}SO_4^{2-}$-labelled proteoglycan (3—8 μg hexuronic acid equivalent/ml or 66—$176 \cdot 10^2$ cpm/ml) was incubated with the cells at 25°C for 90 min and the cellular binding or uptake of labelled proteoglycan was estimated. The results summarized in Table III indicate that the labelled proteoglycan of an amount corresponding to 2—3 μg hexuronic acid/10^9 cells is firmly bound to or taken up by the cells. It may be noted that the cellular binding of proteoglycan as observed in the present study seems rather close to the amount of cell surface proteoglycan in the normal liver cells: i.e., 10^9 liver cells usually give plasma membranes equivalent to 5 mg protein, and liver plasma membranes may contain approx. 0.8 μg hexuronic acid/mg protein.

Discussion

Recently, several groups of investigators [24—27] tried to isolate proteoglycans of large molecular weight from plasma membranes of various tissues. These proteoglycan preparations were susceptible to β-elimination similar to the proteoglycan preparation from liver plasma membranes in the present study, suggesting that acid mucopolysaccharide chains are linked to a protein moiety via O-glycosidic linkages.

In the present study we have shown that purified preparations of proteoglycan from rat liver plasma membranes dose-dependently inhibit the concanavalin A-mediated agglutination of trypsinized or untrypsinized AH-130F(N) cells (Fig. 5a—c). Moreover, the inhibitory effect of proteoglycan was shown to be retained in the cells even after an excess of proteoglycan molecules was removed from the preincubate (Fig. 6), suggesting that some persistent alterations occurred in the cell surface architecture due to the externally-added proteoglycan. The result seems to be supported also by the fact that significant amounts of $^{35}SO_4^{2-}$-labelled proteoglycan are retained by the cells preincubated with the labelled proteoglycan even after repeated washing (Table III).

The possibility that the inhibitory effect of proteoglycan on concanavalin A-mediated agglutination may be due to the contamination of some concanava-

lin A-binding glycoproteins seems unlikely mainly from the following reasons: (a) sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the proteoglycan preparations (stained with fluorescamine) did not give any fluorescent bands other than the two (major and minor) bands which are also stained with Alcian blue; (2) proteoglycan which was passed through a column of concanavalin A-Sepharose (sample B) was as effective as sample A; (3) hexose contents of proteoglycan preparations (samples A and B) were negligibly small; (4) trypsinized AH-130F(N) cells preincubated with sample A became resistant to concanavalin A-mediated agglutination even after an excess of proteoglycan was removed from the medium.

Similarly, the possibility that the inhibitory effect of proteoglycan should be ascribed solely to the interactions of the acid mucopolysaccharide moiety of proteoglycan with concanavalin A molecules [28,29] seems less plausible because the alkali-treated sample A (Fig. 5a), the acid mucopolysaccharide preparation from papain-treated plasma membranes (Table II) and the reference preparation of heparan sulfate (Table II) were less effective than the intact proteoglycan preparations.

These results indicate that not only the acid mucopolysaccharide (heparan sulfate) moiety but also the protein moiety in a proteoglycan molecule are required for the complete activity, suggesting that the protein moiety, which is probably hydrophobic, may be useful for anchoring the molecules on the surface of host cells.

The ascites hepatoma cells, as have been reported in an earlier paper, possess their own proteoglycans on the cell surfaces although the nature of acid mucopolysaccharide moieties is different from that of normal liver cells [9]. The proteoglycan molecules from normal liver plasma membranes, when introduced to an ascites hepatoma cell suspension (trypsinized or untrypsinized), may be firmly taken up by the cells, forming a cell coat-like structure similar to the cell coat of normal liver cells on the host cell surface.

The polyanionic acid mucopolysaccharide chains of proteoglycans with hydrophobic protein cores anchored in the lipid bilayer of plasma membranes may form a gel-like structure on the cell surface due to the cross-linkages via Ca^{2+} and the electrostatic repulsion among the anionic groups. The presence of such network structure of proteoglycans on the cell surface may interfere with the lateral movement of lectin-bound glycoproteins on the cell surface, resulting in the inhibition of lectin-mediated agglutination of cells. Conversely, the removal of acid mucopolysaccharide moieties or the decomposition of gel-like structure over the cell surface by mild protease treatment may facilitate the lectin-induced cell agglutination not only in the untransformed cells but also in the transformed ones.

Acknowledgement

The present study was supported in part by the Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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