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A Fibroblast Heparan Sulphate Proteoglycan with a 70 kDa Core Protein is Linked to Membrane Phosphatidylinositol

ARTUR SCHMIDTCHEN*, ROGER SUNDLER and LARS-ÅKE FRANSSON

Department of Physiological Chemistry, University of Lund, P.O.B. 94, S-221 00, Lund, Sweden

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Here we present evidence that a fibroblast heparan sulphate proteoglycan of approx. 300 kDa and with a core protein of apparent molecular mass 70 kDa is covalently linked to the plasma membrane *via* a linkage structure involving phosphatidylinositol. Phosphatidylinositol-specific phospholipase C releases such a heparan sulphate proteoglycan only from cells labelled with [^{35}S]sulphate in the absence of serum. Cell cultures labelled with [^{3}H]*myo*-inositol in the absence or presence of serum produce a radiolabelled heparan sulphate proteoglycan which was purified by gel-permeation chromatography and ion-exchange chromatography on MonoQ. Digestion with heparan sulphate lyase and analysis by gel-permeation chromatography and sodium dodecylsulphate-polyacrylamide gel-electrophoresis revealed that the ^{3}H-label is associated with a core protein of apparent mass 70 kDa.

Cell surface proteins are either bound peripherally to the polar surface of the plasma membrane or they penetrate the phospholipid bilayer by way of a hydrophobic portion of the polypeptide. In recent years, it has become evident that proteins can also be anchored to the membrane *via* a covalent linkage to phosphatidylinositol [1-3]. Some 30-40 such proteins have now been tentatively identified and they include enzymes, antigens, cell adhesion molecules and receptors [4]. All appear to reside on the extracellular side of the plasma membrane and in most cases identification has been based on their susceptibility to phosphatidylinositol-specific phospholipase C. Another means of identification is to use anchor-specific radiolabelled precursors such as inositol [2, 5].

Membrane proteins can also contain covalently attached linear chains of glycosaminoglycans forming proteoglycans [6, 7]. Proteoglycans are also associated with the cell surface as peripheral or integral plasma membrane components [8]. Studies by Ishihara *et al.* [9] show that some heparan sulphate proteoglycans in a rat hepatocyte cell line may be anchored to the plasma membrane *via* a phosphatidylinositol moiety. It was proposed that insulin-

*Author for correspondence

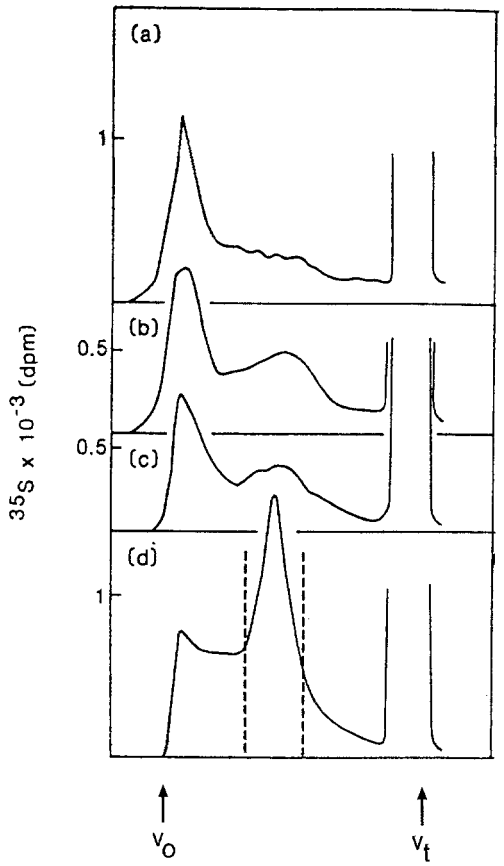

Figure 1. Gel chromatography on Sepharose CL-4B of [^{35}S]sulphate-labelled proteoglycans released from cultured fibroblasts by phosphatidylinositol-specific phospholipase C. Skin fibroblasts were grown to confluence in 10 cm^2 dishes using the regular growth medium. Confluent cells were incubated with sulphate-deficient medium for 1 h at 37°C and then with 50 μCi/ml of [^{35}S]sulphate in the same medium for 24 h in the presence (a-b) or absence (c-d) of serum. The medium was decanted, the cells were washed with 1 ml of phosphate-buffered saline (37°C) and then treated at 37°C for 10 min with 1 ml of serum-free sulphate-deficient medium containing (a and c) no further additions, (b and d) 10 μg of phosphatidylinositol-specific phospholipase C and applied to the column. Material as indicated by the dashed lines in (d) was pooled. V_o, void volume; V_t, total volume.

activated endogenous phospholipase C can cleave the linkage between the core protein-attached inositol and the membrane lipid releasing a proteoglycan with a *myo*-inositol-phosphate moiety still attached to its core protein. More recent studies indicate that rat ovarian granulosa cells [10] and rat Schwann cells [11] also produce a subpopulation of heparan sulphate proteoglycans anchored *via* phosphatidylinositol to the cell membrane.

We, and others, have previously identified multiple forms of heparan and chondroitin/dermatan sulphate proteoglycans in various types of fibroblasts [12-15]. Human skin fibroblasts produce a number of heparan sulphate proteoglycans, with core proteins of apparent molecular masses 350, 250, 130, 90, 70, 45 and possibly 35 kDa as well as chondroitin/dermatan sulphate proteoglycans with core proteins of 400-500, 90 and 45 kDa [15]. The two proteoglycans with 90 kDa core proteins, one substituted with heparan sulphate and the other with chondroitin/dermatan sulphate are exclusively cell-associated. Heparan sulphate proteoglycan with a 70 kDa core can be identified both in the cell layer and in the medium. In this report we present evidence that heparan sulphate proteoglycan with a core protein of 70 kDa is linked to the plasma membrane *via* phosphatidylinositol.

Experimental

Materials

The radioactive materials, $Na_2{}^{35}SO_4$ (1310 Ci/mmol), *myo*-[2-^{3}H]inositol (12.8 Ci/mmol), [^{3}H]leucine (50 Ci/mmol), were purchased from the Radiochemical Centre, Amersham, Bucks., UK. The phosphatidylinositol-specific phospholipase C was purified from *Bacillus cereus* [16]. Sepharose CL-4B, Sephacryl S500HR and Mono-Q HR 5/5 were from Pharmacia-LKB, Uppsala, Sweden; inositol hexaphosphate (sodium phytate) from BDH, Poole, UK; and other reagents from sources listed previously [15].

Culture Conditions

Cultures of human embryonic skin fibroblasts were established and grown in Earles MEM supplemented with 10% (v/v) donor calf serum, 2 mM glutamine, penicillin (100 units/ml) and streptomycin (100 μg/ml) as described previously [15]. When cells were incubated with $^{35}SO_4$ (50 μCi/ml) or with [^{3}H]leucine a low sulphate/low leucine medium was used and incubation with *myo*-[^{3}H]inositol (50 μCi/ml) and [^{35}S]sulphate (5 μCi/ml) was conducted in medium 199. Labelling was usually performed without serum for 24-48 h.

Proteoglycan Extraction and Isolation

After incubation with radiolabelled precursors, the culture medium was decanted and the cell layer was carefully washed three times with ice-cold phosphate-buffered saline (1-5 ml; 0.137 M NaCl/3 mM KCl/8 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4). The cell layer was extracted with 4 M guanidine-HCl/1% (by vol) Triton X-100/50 mM sodium acetate buffer, pH 5.8 containing *N*-ethyl-maleimide (10 mM), EDTA (10 mM) and di-isopropyl phosphorofluoridate (1 mM). The extract was clarified by centrifugation and stored frozen until use.

Density Gradient Ultracentrifugation in CsCl

After labelling with [^{3}H]leucine the guanidine/Triton extract of the cell layer was adjusted to a density of 1.35 g/ml in 4 M guanidine-HCl/1% Triton X-100 by adding solid CsCl, Triton X-100 and water [15]. Centrifugation was performed at 36,000 rev./min for 65 h at 15°C, in

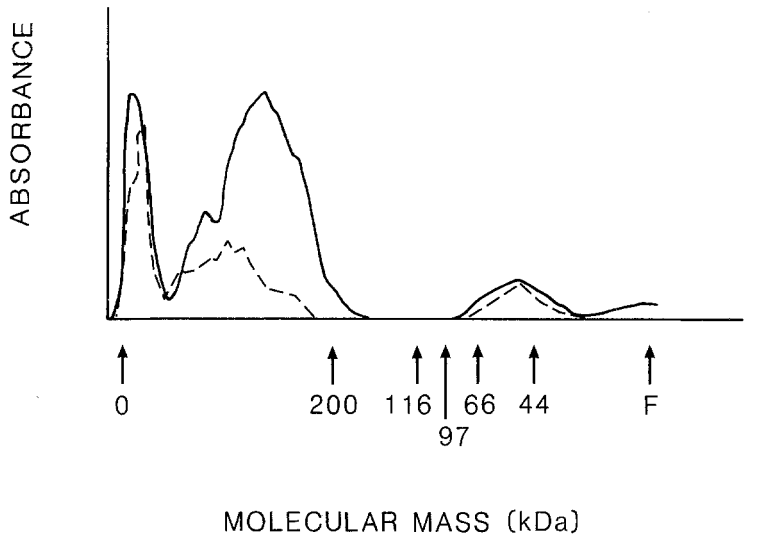

Figure 2. SDS/polyacrylamide gel-electrophoresis of phospholipase-released [^{35}S]sulphate-labelled proteoglycans. Material derived from untreated (- - -) and treated (—) cells (c and d in Fig. 1 respectively) was further examined by SDS/polyacrylamide electrophoresis (3-12% gels) in the presence of β-mercaptoethanol. The gel was then subjected to fluorography followed by scanning densitometry. 0, origin; F, front.

a Beckman model L8 60 M ultracentrifuge with a Ti 50.2 rotor. Material with a density over 1.3 g/ml was collected by emptying the tubes from the bottom, and concentrated by ultrafiltration in a Novacell 10 K (Filtron).

Chromatographic Procedures

Gel permeation chromatography was carried out on columns (0.6 x 150 cm) of Sepharose CL-4B or on Sephacryl S500HR (1.0 x 100 cm or 2.5 x 100 cm) which were eluted with 4 M guanidine-HCl/0.2% (by vol) Triton X-100/50 mM sodium acetate buffer, pH 5.8 containing ovalbumin (5 μg/ml). Recovery was 80% or better in all cases.

Ion-exchange chromatography on Mono-Q HR was performed on a Mono-Q HR 5/5 column connected to an f.p.l.c. system (LKB) as previously described [15]. Samples contained heparan sulphate carrier (20-200 μg) and were dialysed against 7 M urea, 0.1% Mulgophene and 10 mM Tris/HCl, pH 8.0. After injection of the sample the column was eluted with the equilibrating buffer at a rate of 0.5 ml/min for 10 min and then with a 60 min linear gradient up to 1.2 M NaCl in the same buffer. Pooled material was concentrated (<2 ml) and stored at -20°C.

Degradative Methods

Purified proteoglycans were dialysed against 0.1% Triton X-100, 3 mM calcium acetate and 10 mM Hepes, pH 7.0. Samples were treated with heparan sulphate lyase in the presence of proteinase inhibitors as described [15].

SDS-Polyacrylamide Gel Electrophoresis

This was performed as described [15] on 3-12% polyacrylamide gels. Samples (approx. 10,000 [^{3}H]dpm) were precipitated with a nine-fold excess of 95% ethanol, centrifuged (4000 x *g* for 60 min), dried and dissolved in 50 µl 5% (w/v) SDS/20% (by vol) glycerol/4 mM EDTA/0.04% Bromophenol Blue/125 mM Tris/HCl, pH 6.8. To reduce disulphide bonds β-mercaptoethanol was added to a final concentration of 10% (by vol). Samples were boiled for 3 min and electrophoresed for approx. 30 h at a constant current of 10 mA. Gels were stained with Coomassie Brilliant Blue R-250, destained and soaked in Amplify NAMP-100 (Amersham). Fluorography was performed for 2-6 weeks, with Kodak XAR-5 film. Scanning was performed with a videodensitometric system developed by Mr. Lars Kopp (Makab, Göteborg, Sweden).

Results

Release of Proteoglycans with Phosphatidylinositol-specific Phospholipase C

Confluent human embryonic skin fibroblasts pre-labelled with radiosulphate in the absence or presence of serum were incubated for 10 min with or without phosphatidylinositol-specific phospholipase C. The incubation media were chromatographed on Sepharose CL-4B (Fig. 1). There was increased release of ^{35}S-proteoglycan upon phospholipase treatment, especially from cells not exposed to serum (cf Fig. 1b and d). There was also a spontaneous release of proteoglycan from untreated cells. These proteoglycans eluted mainly at K_{av} 0-0.25 (Fig. 1a-d). The proteoglycan released by phospholipase from cells pre-labelled in the absence of serum eluted with a K_{av} of 0.4 (Fig. 1d). This proteoglycan material was pooled, treated with heparan sulphate lyase and re-chromatographed on the same column. About 90% of the label was found in the total volume (results not shown) showing that most of the proteoglycans were heparan sulphate-containing ones. In a parallel experiment proteoglycans corresponding to those analysed in Fig. 1c and d were subjected to SDS-polyacrylamide gel electrophoresis (Fig. 2). The apparent molecular mass of the phospholipase-released proteoglycan was estimated to about 300 kDa (Fig. 2). The spontaneously released proteoglycans were of higher molecular mass. No proteoglycan from either serum-exposed or non-exposed cells could be released with 50 mM inositol-hexaphosphate [9].

Isolation and Characterization of [^{3}H]Inositol- and [^{35}S]Sulphate- or [^{3}H]Leucine-labelled Heparan Sulphate Proteoglycans

The guanidine-Triton extract from [^{3}H]leucine-labelled cultures (no serum) was subjected to isopycnic density gradient centrifugation. Material with a density higher than 1.3 mg/ml was collected [15], concentrated and chromatographed on a column (2.5 x 100 cm) of

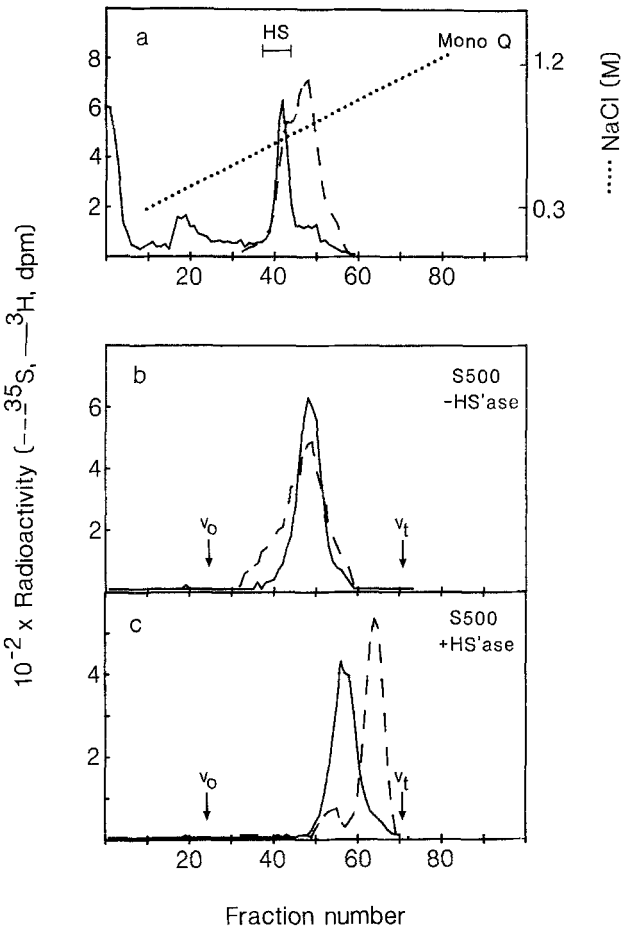

Figure 3. Ion-exchange h.p.l.c. on Mono-Q (a) and gel-permeation chromatography on Sephacryl S-500 HR (b and c) of [^{3}H]inositol- and [^{35}S]sulphate-labelled proteoglycans. Proteoglycans produced in the absence of serum were isolated from the cell extract after preparative gel permeation chromatography (see the Experimental section) and subjected to ion-exchange chromatography on Mono-Q (a). Heparan sulphate-containing proteoglycans were pooled as indicated by the bar (HS), and chromatographed on a column (1 x 100 cm) of Sephacryl S500 HR before (b) and after treatment with heparan sulphate lyase (c). v_o, void volume; v_t, total volume.

Sephacryl S500 HR. The extract from [^{3}H]inositol and [^{35}S]sulphate labelled cells (no serum) was directly chromatographed on the same column. Material corresponding to large (K_{av} 0.1-0.4) and small (K_{av} 0.4-0.8) proteoglycans was pooled and subjected to ion-exchange chromatography on Mono-Q as previously described [15]. In the pool containing large proteoglycans no [^{3}H]inositol-labelled material co-chromatographed with the [^{35}S]sulphate-labelled material and this fraction was therefore not analysed further (results not shown). The small proteoglycans consisted of material that eluted from Mono-Q as a broad [^{35}S]sulphate-

labelled peak between 0.7-1.0 M NaCl (Fig. 3a). [^{3}H]Inositol-labelled material eluted only in the position corresponding to heparan sulphate proteoglycans (Fig. 3a, bar). This material was pooled, concentrated and dialysed against digestion buffer (see the Experimental section) and subjected to gel permeation chromatography on S500 HR before and after digestion with heparan sulphate lyase (Fig. 3b and c). Prior to digestion the [^{3}H]inositol- and [^{35}S]sulphate-labelled proteoglycans essentially co-chromatographed (Fig. 3b). After enzymatic removal of the heparan sulphate side chains (Fig. 3c) the [^{3}H]inositol-labelled core protein emerged in a more retarded position and ahead of the [^{35}S]sulphate-labelled glycosaminoglycan chain-fragments.

In another experiment the core proteins of [^{3}H]leucine-labelled heparan sulphate proteoglycans were compared with those derived from the [^{3}H]inositol-labelled heparan sulphate proteoglycans. Before and after treatment with heparan sulphate lyase the samples were subjected to SDS/polyacrylamide-gel electrophoresis on 3-12% gels in the presence of β-mercaptoethanol. As seen in Fig. 4a (dashed line) undigested [^{3}H]leucine-labelled heparan sulphate proteoglycans migrated primarily as a broad band around 200-300 kDa. Digestion with heparan sulphate lyase afforded several core proteins with apparent molecular masses of 130, 90, 70, 45 and 35 kDa. A 35 kDa component also appeared after reduction of the intact proteoglycans (Fig. 4a, dashed line). This component was not seen in unreduced and undigested samples (results not shown) [15].

Digestion of [^{3}H]inositol-labelled heparan sulphate proteoglycans with the same enzyme (Fig. 4b) yielded core proteins with apparent molecular masses of 70 kDa (see arrow) and approx. 35-40 kDa under reduced conditions. No 35 kDa [^{3}H]inositol-labelled component was seen after reduction of intact proteoglycan (Fig. 4b, dashed line). Some material streaking around 50 kDa was apparently not sensitive to heparan sulphate lyase. All the results concerning [^{3}H]inositol-incorporation were essentially identical irrespective of whether the cells were labelled in the absence or presence of serum (results not shown).

Discussion

Skin fibroblasts appear to synthesize a subpopulation of heparan sulphate proteoglycans that are anchored to membrane phosphatidylinositol. This is supported by the following findings, (a) cell-surface-associated heparan sulphate proteoglycan from confluent cultures maintained under serum-free conditions can be released by brief treatment with phosphatidylinositol-specific phospholipase C, (b) incubation of cells with [^{3}H]inositol results in the formation of ^{3}H-labelled heparan sulphate proteoglycan and (c), the ^{3}H-isotope is associated with a protein core of approx. 70 kDa.

According to the model described for hepatocytes [9] activation of endogenous phospholipase by serum or insulin should convert the membrane-bound proteoglycan to a non-covalently bound form. Furthermore, serum contains a phosphatidylinositol-specific phospholipase D-like activity capable of releasing inositol-bound ectoproteins [17]. Therefore, it is not surprising that serum-exposed fibroblasts seem to lack phospholipase C-sensitive proteoglycans.

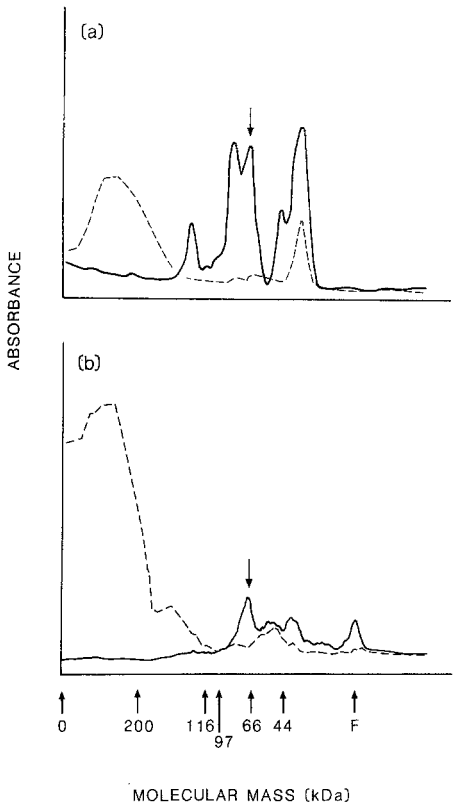

Figure 4. SDS/polyacrylamide gel-electrophoresis of cell-associated heparan sulphate proteoglycans labelled with [^{3}H]leucine (a) or with [^{3}H]inositol and [^{35}S]sulphate (b). Isolated heparan sulphate proteoglycans (see the Experimental section) were analysed by SDS/polyacrylamide gel-electrophoresis (3-12%) in the presence of β-mercaptoethanol. The gels were scanned after fluorography for 2-6 weeks. The scans are: (a) [^{3}H]leucine-labelled proteoglycans and (b) [^{3}H]inositol and [^{35}S]sulphate labelled proteoglycans before (- - -) and after (—) digestion with heparan sulphate lyase. Arrow indicates the position of the 70 kDa core protein. In (b) the undigested proteoglycan contains both ^{35}S and ^{3}H, whereas the core proteins only contain ^{3}H.

^{3}H-Label from *myo*-[2-^{3}H]inositol was incorporated into the 70 kDa core protein of a heparan sulphate proteoglycan. We have not identified the incorporated ^{3}H-label as inositol but (a) conversion of inositol to other metabolites have not been found in fibroblasts, (b)

insignificant amounts of isotope was found in other, more abundant proteoglycan fractions and (c), [^{3}H]inositol was incorporated into only 7-8 other cell/matrix-associated proteins (Schmidtchen, unpublished results). Therefore, conversion of [^{3}H]inositol to general carbohydrate precursors seems highly unlikely.

[^{3}H]Leucine-labelled small heparan sulphate proteoglycans contain multiple core proteins with apparent molecular masses of 130, 90, 70, 45 and possibly 35 kDa [15]. The 35 kDa component is only seen after treatment with β-mercaptoethanol and may therefore be the result of cleavage of a core protein of 70 kDa that consists of two 35 kDa domains connected by interchain disulphide bonds. The appearance and intensity of the 35-40 kDa band on reduced gels varies with different preparations and could be due to variable proteolytic scission of the 70 kDa core protein [15]. A 35 kDa [^{3}H]leucine-labelled glycan-free component was released after reduction of intact proteoglycan. However, a corresponding [^{3}H]inositol-labelled component was not seen. Therefore, it is likely that the heparan sulphate side-chains and the [^{3}H]inositol are located to the same 35 kDa domain.

The heparan sulphate proteoglycan with a 70/35 kDa core protein is synthesized by serum-exposed cells [15] and, as shown here, it also incorporates [^{3}H]inositol in the presence of serum. However, a ^{35}S-labelled proteoglycan produced by serum-exposed cells could not be released by phospholipase C or inositol hexaphosphate. This could be due to other interactions with the cell surface or matrix components [8] or modifications of the glycolipid anchor [3].

The heparan sulphate proteoglycan from rat ovarian granulosa cells [10] has a major core protein with an apparent molecular mass of 80 kDa and a smaller one with an apparent molecular mass of 30-40 kDa under reducing conditions. Glycolipid-linked proteoglycans produced by rat Schwann cells [11] and hepatocytes [9] also seem to be of the same size and it is therefore possible that there is a family of glycolipid-anchored membrane-associated heparan sulphate proteoglycans in many cell types, including human fibroblasts [12-15]. The functions of these proteoglycans are still unknown but it is possible that anchoring to the cell surface *via* a phosphatidylinositol group may confer new properties to proteins or proteoglycans, such as (a) increased mobility in the cell membrane, (b) controlled release from the cell surface by the action of endogenous cell surface-bound phospholipases [2, 18] or (c) targeting of molecules to apical parts of cells [19]. Further studies on the heparan sulphate proteoglycan described here are needed to determine the structure of the inositol-containing glycolipid anchor and its constituents as well as the metabolism of the proteoglycan in relation to different growth conditions.

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