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Plasma-membrane-intercalated heparan sulphate proteoglycans in an osteogenic cell line (UMR 106-01 BSP)

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The heparan sulphate (HS) proteoglycans associated with the cell layer of a rat osteosarcoma cell line [UMR 106-01 (BSP)] were compared with similar cell-associated proteoglycans from other cells, and their interaction with the plasma membrane was studied. HS proteoglycans were metabolically labelled by incubation of cell cultures with [^{3}H]glucosamine or [^{3}H]leucine and [^{35}S]sulphate. HS proteoglycan core protein preparation generated by heparitinase digestion of the major species from UMR 106-01 (BSP) cells co-migrated on PAGE with identical preparations from ovarian granulosa cells and parathyroid cells (at ~ 70 kDa). The hydrophobic nature of the major HS proteoglycans from these diverse cell lines, based on elution position from octyl-Sepharose, were also comparable. Linkages of the HS proteoglycan to the cell membrane were investigated by labelling plasma-membrane preparations with a lipid soluble photoactivatable reagent, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine (TID), which selectively labels plasma-membrane-spanning peptide domains. Purified HS proteoglycan from UMR 106-01 (BSP) cells was shown to be accessible to the [^{125}I]TID, and the core protein portion of the molecule was labelled, confirming its close association with the plasma membrane. Approx. 36% of ^{35}S-labelled HS proteoglycans were released from the cell surface by phospholipase C (*Bacillus thuringiensis*), which specifically cleaves phosphatidylinositol-linked proteins. In the presence of insulin, the metabolism of the phospholipase C-sensitive population was unaltered; however, release of the phospholipase C-insensitive population into the medium was increased. These data indicate that a subpopulation of HS proteoglycans are covalently bound to the plasma membrane by a glycosylphosphatidylinositol structure, with the remainder representing those species directly inserted into the plasma membrane via a hydrophobic peptide domain. These observations are similar to those reported for ovarian granulosa cells [Yanagishita & McQuillan (1989) *J. Biol. Chem.* **264**, 17551–17558], and thus may represent a general phenomenon for many cell types.

INTRODUCTION

The osteogenic sarcoma cell line UMR 106, and the subclone UMR 106-01, were derived originally from a transplantable rat osteogenic sarcoma, and have been widely used in the study of osteoblast phenotype and bone metabolism *in vitro* [1–3]. Recently we demonstrated that a clonal variant of the UMR 106-01 lineage synthesized and secreted elevated levels of a highly sulphated form of bone sialoprotein [4], and this cell line has therefore been designated UMR 106-01 (BSP) (BSP = bone sialoprotein). We subsequently characterized the spectrum of proteoglycans synthesized by the UMR 106-01 (BSP) cells [5], and demonstrated that it was consistent with an osteoblast phenotype. Of particular interest, in addition to the high expression of BSP, was the marked expression of a cell-layer-associated heparan sulphate proteoglycan which we postulated was related to similar species isolated from such diverse cell lines as ovarian granulosa cells [6] and parathyroid cells [7].

Heparan sulphate proteoglycans have been observed to be associated with the surface of many cell types [8] although, with few exceptions [9], they have not previously been reported to be associated with osteoblasts. A large proportion of heparan sulphate proteoglycans on cell surfaces are transmembrane molecules. Although many of their functions remain unclear, these heparan sulphate proteoglycans appear to be involved in both cell–cell and cell–matrix interactions [8], and also to act as potential growth-factor binders [10]. With the exception of syndecan [11] and fibroglycan [12], evidence for membrane intercalation has largely been based upon indirect criteria, including the necessity for detergents in extraction [13], the binding to hydrophobic matrices [14,15] and intercalation into artificial micelles [16]. We recently demonstrated, by use of a hydrophobic photoactivatable probe, that the heparan sulphate proteoglycans associated with the cell membrane of ovarian granulosa cells consist of two distinct populations: one that is directly intercalated into the plasma membrane and a second intercalated via a linkage structure involving phosphatidylinositol [17]. Previously Ishihara *et al.* [18] had shown the presence of phosphatidylinositol-linked heparan sulphate proteoglycans on hepatocytes. Phospholipase-releasable heparan sulphate proteoglycan has also been found in Schwann cells [19] and embryonic heart cells [20], and recently a heparan sulphate proteoglycan with a proposed phosphatidylinositol linkage to the cell membrane has been cloned [21].

The presence of heparan sulphate has not been shown in extracts of intact bone [22], and thus has largely been ignored in osteoblast cultures. However, whereas matrix proteoglycans (decorin and biglycan) may accumulate in the osteoid, membrane-associated proteoglycans are likely to have much shorter half-lives and would thus not comprise a significant portion of the non-collagenous milieu. It is also likely that methods designed to optimize recovery of matrix components would not be well suited to the isolation of these highly hydrophobic molecules. In the present study we have extended our initial identification of the UMR 106-01 (BSP) heparan sulphate proteoglycan to define its membrane localization, its relationship with other heparan sulphate proteoglycans characterized previously in our laboratory, and its linkage to the cell layer.

Abbreviations used: BSP, bone sialoprotein; MEM, Eagle's minimum essential medium; [^{125}I]TID, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)diazirine; PLC, phosphatidylinositol-specific phospholipase C; GPI, glycosylphosphatidylinositol.

EXPERIMENTAL

Materials

[^{125}I]TID (10 Ci/mmol) was purchased from Amersham Corp.; [^{35}S]sulphate (~ 1.0 mCi/mmol), D-[6-^{3}H]glucosamine hydrochloride (20–38 Ci/mmol), L-[3,4,5-^{3}H]leucine (140 Ci/mmol), and ENTENSIFY from du Pont–New England Nuclear; Q-Sepharose Fast Flow, octyl-Sepharose CL-4B, prepacked Superose 6, Sephadex G-50 (fine grade), and Percoll from Pharmacia LKB Biotechnology; guanidinium chloride, urea, ^{14}C-labelled protein standards and phenylmethanesulphonyl fluoride from Life Technologies/Bethesda Research Laboratories; Triton X-100 from Pierce Chemical Co.; CHAPS from Behring Diagnostics; chondroitinase ABC (*Proteus vulgaris*) and heparitinase (*Flavobacterium heparinum*) from Seikagaku America; 6-aminohexanoic acid, benzamidine, and X-Omat AR film from Eastman Kodak Co.; electrophoresis chemicals from Bio-Rad; Ready Safe liquid scintillation cocktail from Beckman; and Eagle's Minimal Essential Medium and fetal-calf serum from Life Technologies/Grand Island Biological Co. Phospholipase C (*Bacillus thuringienis*) was generously given by Dr. M. G. Low (Columbia University, New York, NY, U.S.A.).

Cell culture

UMR 106-01 (BSP) cells, a clonal variant enriched in the synthesis of BSP has been described [4,5]. Cells were cultured and passaged as described by Forrest *et al.* [1]. Frozen cells were stored at −80 °C in 10% (v/v) dimethyl sulphoxide/fetal-calf serum. Cells were cultured in Eagle's minimum essential medium (MEM) containing added non-essential amino acids, 20 mM-Hepes, pH 7.2, gentamicin sulphate (50 μg/ml) and 10% (v/v) fetal-calf serum in 75 cm^2 flasks at 37 °C in a humidified 5% CO_2 atmosphere.

Subculturing to 75 cm^2 flasks for passaging or to 35 or 150 mm-diameter culture dishes for labelling experiments was accomplished by incubating confluent cell layers with 0.05% (w/v) trypsin/0.53 mM-disodium EDTA in Hanks balanced salt solution without Ca^{2+} and Mg^{2+} at 37 °C for 5 min. Cell cultures (~ 1×10^8 cells/150 mm dish) were metabolically labelled either with [^{3}H]leucine (100 μCi/ml) and [^{35}S]sulphate (30 μCi/ml) or [^{3}H]glucosamine (50–100 μCi/ml) and [^{35}S]sulphate (50–100 μCi/ml) for 20 h at 37 °C in a total volume of 10 ml. After labelling, each cell was washed three times with MEM containing heparin (1 mg/ml) at 37 °C for 30 min. Some of the cell cultures were further treated with 10 ml of trypsin (10 μg/ml) for 15 min at 37 °C to remove cell-surface and pericellular proteoglycans [5]. Cell-associated proteoglycans were extracted with 4 M-guanidinium chloride/50 mM-sodium acetate, pH 6.0, containing 2% Triton X-100 and proteinase inhibitors (5 mM-*N*-ethylmaleimide, 0.5 mM-phenylmethanesulphonyl fluoride, 0.1 M-6-aminohexanoic acid, 10 mM-benzamidine hydrochloride and 10 mM-EDTA).

Photoaffinity labelling with [^{125}I]TID

After 20 h labelling of confluent UMR 106-01 (BSP) cells with [^{35}S]sulphate (20 μCi/ml) and [^{3}H]leucine (100 μCi/ml), cell cultures were washed three times with 0.25 M-sucrose/50 mM-sodium phosphate, pH 7.4 at 0 °C, and scraped from the dishes with a rubber policeman in a final volume of 2 ml of the same sucrose buffer. The cell suspension was homogenized with a Potter–Elvehjem homogenizer. A plasma-membrane-enriched fraction was prepared by centrifugation of the homogenate in a Percoll gradient. Briefly, 1 ml of homogenate was overlaid on 4 ml of 20% (v/v) Percoll solution in the homogenization buffer, and centrifuged at 12000 rev./min for 1 h at 4 °C in a Beckman SW Ti-55 rotor. The majority of the plasma membrane was recovered in a low-density fraction near the top of the Percoll gradient. The plasma-membrane-enriched fraction was mixed with 300–500 μCi of [^{125}I]TID in about 50 μl of ethanol in a final volume of 0.5–1.0 ml and incubated for 30 min with mixing at 0 °C in the dark. After partitioning of the TID into the hydrophobic plasma membrane, samples in quartz cells were irradiated with long-wavelength u.v. light at the shortest range for 10 min. After irradiation, samples were solubilized with 8 M-formamide/0.4 M-NaCl/50 mM-sodium acetate, pH 6.0, containing 2% Triton X-100 and proteinase inhibitors. The extract was applied directly to a 2 ml column of Q-Sepharose (0.7 cm × 5 cm) equilibrated with 8 M-formamide/0.4 M-NaCl/50 mM-sodium acetate/0.5% Triton X-100, pH 6.0. After extensive washing of the column with the same solvent (until ^{125}I radioactivity in the washes was negligible), bound macromolecules were eluted with 4 M-guanidinium chloride/0.5% Triton X-100/50 mM-sodium acetate, pH 6.0.

^{125}I radioactivity was measured either directly with a Beckman Gamma 4000 counter or with a Beckman LS 5801 instrument using liquid-scintillation-counting techniques. ^{3}H, ^{35}S and ^{125}I radioactivites were differentiated by appropriate window settings and spill-over corrections on the Beckman LS 5801 instrument.

Purification of labelled proteoglycans

Proteoglycans from extracts of cells labelled with [^{35}S]sulphate and [^{3}H]glucosamine were isolated as described previously [5]. Briefly, the macromolecular fraction was separated from unincorporated radioactive precursors and other salts by chromatography on Sephadex G-50 (fine grade) columns equilibrated and eluted with 8 M-formamide/0.2 M-NaCl/0.5% Triton X-100/50 mM-sodium acetate, pH 6.0. Labelled macromolecules were applied to columns of Q-Sepharose (0.7 cm × 5 cm) pre-equilibrated in the same buffer. Bound material was step-eluted with 4 M-guanidinium chloride/0.5% CHAPS/50 mM sodium acetate, pH 6.0, and concentrated by Centricon-10 ultrafiltration to a final volume of about 0.25 ml. The sample was chromatographed on a column of Superose 6 in 8 M-formamide/0.2 M-NaCl/0.5% Triton X-100/50 mM sodium acetate, pH 6.0. The fractions containing intact heparan sulphate proteoglycans [5] were pooled and applied directly to a column of Q-Sepharose as described above, and the bound proteoglycans were eluted with a continuous NaCl gradient (0.2–1.2 M) with a total volume of 50 ml. The proteoglycans isolated were further analysed as described in the Results section.

Analytical column chromatography

Prepacked Superose 6 (1 cm × 30 cm) was eluted with 4 M-guanidinium chloride/50 mM-sodium acetate/0.5% Triton X-100, pH 6.0, at a flow rate of 0.4 ml/min, and fractions (0.4 ml each) were collected for analyses. The column was calibrated with protein molecular-mass standards. An octyl-Sepharose CL-4B column (0.7 cm × 5 cm) was equilibrated in 4 M-guanidinium chloride/50 mM-sodium acetate, pH 6.0 [23]. Samples were prepared in the same solvent and, immediately before sample application, the column was pre-blocked with 100 μg of BSA [23]. After sample application, a gradient of Triton X-100 (0–0.5% with a total volume of 50 ml) was applied in the same 4 M-guanidinium chloride solvent. Fractions (1 ml each) were collected for analyses. Triton X-100 concentration in the effluent was monitored by u.v. absorbance at 280 nm.

SDS/PAGE

Polyacrylamide linear gradient (4–17%, w/v) slab gels (0.1 cm × 8 cm × 10 cm) and uniform slab gels with different acrylamide concentrations (0.1 cm × 5 cm × 7 cm) were prepared in the buffer system of Laemmli [24], with a stacking gel of 3.5%

polyacrylamide. Electrophoresis was done until the tracking dye was about 1 cm from the bottom. Fluorography was with ENTENSIFY, which was used according to the manufacturer's instructions.

Enzyme digestion of purified proteoglycans

Chondroitinase ABC (100 munits/ml) digestions were done in 0.1 M-Tris/0.1 M-acetate, pH 7.3, containing 10 mM-EDTA, 10 mM-*N*-ethylmaleimide and 5 mM-phenylmethanesulphonyl fluoride for 1.5 h at 37 °C in a final volume of 0.25–0.5 ml. Heparitinase (10 munits/ml) digestions were done in the same buffer, except that 1 mM-$CaCl_2$ was substituted for the EDTA.

Treatment of cell culture with phospholipase C

UMR 106-01 (BSP) cells at 70 % confluency were metabolically labelled with [^{35}S]sulphate (10 μCi/ml) for 20 h. After labelling, cells were washed three times with MEM and chased for 1 h to eliminate BSP from the cell layer (pre-chase period). During the final 30 min of the pre-chase period, some cultures were incubated with phosphatidylinositol-specific phospholipase C (PLC, 0.2 unit/ml) to remove glycosylphosphatidylinositol (GPI)-anchored proteoglycans, and the culture medium collected. Cultures treated or not with PLC were subsequently chased for 9 h in fresh medium (preliminary experiments showed that neither longer incubation time nor addition of fresh enzyme increased the release of proteoglycans, indicating that the PLC digestion was complete). After the chase, media were removed and cell cultures were sequentially treated with PLC (0.2 unit/ml) for 30 min and, after removing the PLC digests, with trypsin (200 μg/ml) for 7 min to quantify GPI-anchored and PLC-resistant cell-surface proteoglycans respectively. The remaining cell layers were extracted with 4 M-guanidinium chloride/50 mM-sodium acetate, pH 6.0, containing 2 % Triton X-100. Each chase medium, enzyme digest and cell extract was analysed by Sephadex G-50 chromatography for total proteoglycan quantification. ^{35}S-labelled macromolecules were further digested with chondroitinase ABC and analysed by Superose 6 chromatography. Cell cultures without chase were similarly analysed. A pulse–chase experiment identical with that described above was carried out in the presence or absence of insulin (10 μg/ml), except that the final chase time was 2 h instead of 9 h.

RESULTS

Comparison of UMR 106-01 (BSP) heparan sulphate proteoglycan core protein with those of ovarian granulosa cells and parathyroid cells

Proteoglycans from confluent cultures of UMR 106-01 cells, parathyroid cells [7] and ovarian granulosa cells [25] were metabolically labelled by incubating cultures in the presence of medium containing [^{3}H]glucosamine (50 μCi/ml) and [^{35}S]sulphate (50 μCi/ml) for 20 h. After removal of the labelling medium, cell layers were washed with medium containing heparin (1 mg/ml) for 30 min at 37 °C, which, in the case of ovarian granulosa cells, has been shown to displace cell-surface proteoglycans which interact with the cell surface by electrostatic forces [6]. Approx. 25 % of the ^{35}S-labelled macromolecules were released from granulosa cells, less than 5 % from UMR 106-01 (BSP) cells, and release from parathyroid cells not determined. The cell layers were then extracted with 4 M-guanidinium chloride/50 mM-sodium acetate, pH 6.0, containing 2 % Triton X-100 and proteinase inhibitors [26]. Cell-layer-associated heparan sulphate proteoglycans were then purified by sequential passage over Sephadex G-50, Q-Sepharose, and Superose 6 columns in combination with specific glycosidases to remove non-heparan sulphate-containing proteoglycans. These isolation procedures were detailed in McQuillan *et al.* [5] for UMR 106-01 (BSP) cells, Yanagishita & McQuillan [17] for granulosa cells, and Yanagishita *et al.* [7] for parathyroid cells. Owing to the striking similarity of the heparan sulphate proteoglycans isolated from these diverse cell lines indicated by chromatographic and biochemical analyses (see the respective papers cited above), the core proteins derived from these proteoglycans were compared by SDS/PAGE. Aliquots of each heparan sulphate proteoglycan preparation, containing similar levels of ^{3}H and ^{35}S, were incubated with and without heparitinase, and portions of both intact and treated samples were analysed on a gradient (4–17 %) SDS/PAGE slab gel (Fig. 1*a*). Intact heparan sulphate proteoglycans from the three cell lines only partially entered the resolving gel under these conditions (lanes 1−, 2− and 3−). However, after heparitinase digestion, the core proteins from the major heparan sulphate proteoglycan of each species were apparent (due to ^{3}H label in the oligosaccharide moieties) and were co-incident for all three preparations at ~ 70 kDa (lanes 1+, 2+, and 3+). In the sample from ovarian granulosa cells (lane 3+) a second band was apparent at a molecular mass of about 35 kDa, which has been observed previously [17], although its relation to the major species remains unclear.

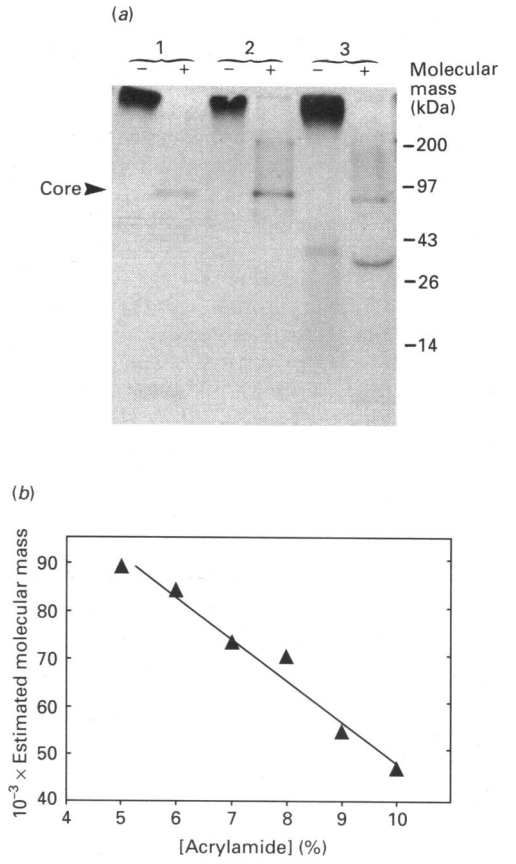


Fig. 1. (*a*) SDS/PAGE/fluorographic analysis of cell-associated heparan sulphate proteoglycans labelled with [^{35}S]sulphate and [^{3}H]glucosamine before (−) and after (+) heparitinase digestion and (*b*) anomalous migration of heparitinase-generated core-protein preparation from UMR 106-01 (BSP) cells on SDS/PAGE

(*a*) 1, UMR 106-01 (BSP) cells; 2, parathyroid cells; 3, ovarian granulosa cells. Molecular-mass markers are indicated. (*b*) Heparan sulphate proteoglycan was labelled as indicated above and analysed by SDS/PAGE using a range of acrylamide concentrations.

Cell-layer-associated heparan sulphate proteoglycans isolated from cultures of skin fibroblasts, hepatocytes and kidney epithelial cell lines (results not shown) also exhibited a similar size of the core protein of the major heparan sulphate proteoglycan. This would suggest that the major cell-associated heparan sulphate proteoglycan in these cell lines is represented by closely related core proteins, even though previous estimates of their core-protein sizes vary widely, from ~ 30 kDa to ~ 200 kDa [8,12,27–29]. One cause for these apparent discrepancies in molecular-mass estimates was identified with a Ferguson analysis [30] of the core protein derived from the UMR 106-01 (BSP) heparan sulphate proteoglycan. After heparitinase treatment, the core protein was analysed on a series of SDS/PAGE gels ranging from 5% up to 10% acrylamide concentration, and the apparent molecular mass of the core protein estimated from its mobility relative to those of globular protein standards. The estimated size varied almost 2-fold with increasing acrylamide concentration (Fig. 1*b*), resolving in the range of 46–87 kDa. Possible reasons for this variability are reviewed elsewhere [30]. However, the results indicate that estimates of proteoglycan core proteins on SDS/PAGE require special precautions. We have previously noted [5,17] that the size of a core protein heavily substituted with oligosaccharides is better estimated by molecular-sieve chromatography in the presence of chaotropic solvents.

Hydrophobicity of the heparan sulphate proteoglycan

The cellular distribution and biochemical similarity of the heparan sulphate proteoglycan from UMR 106-01 (BSP) cells to that isolated from both granulosa cells and parathyroid cells strongly suggested that it, too, may be intercalated in a hydrophobic membrane. Heparan sulphate proteoglycans purified from cell-layer extracts by ion-exchange chromatography were further analysed by octyl-Sepharose chromatography in 4 M-guanidinium chloride eluted with a gradient of 0–0.5% Triton X-100 [23] (Fig. 2). Two major ^{35}S-labelled peaks were apparent, one in the unbound fraction (~ 30% of the total) and one at the inflection point at 0.18% Triton X-100 (~ 65% of the total). Similar chromatographic profiles (results not shown) were obtained for heparan sulphate proteoglycans from both ovarian granulosa cells and parathyroid cells, with the amounts of ^{35}S activity eluted at 0.18% Triton X-100 being ~ 45% and ~ 54% respectively.

The unbound peak from octyl-Sepharose was eluted late on Superose 6, in the range expected for small proteins and glycosaminoglycan chains not associated with an intact core protein, indicating that it represents primarily intracellular degradation products. The peak which was eluted at the inflection point in the Triton X-100 gradient eluted at K_d of 0.17 (~ 150 kDa) on Superose 6, and thus represents a highly hydrophobic intact heparan sulphate proteoglycan. We obtained similar data for ovarian granulosa cells [17].

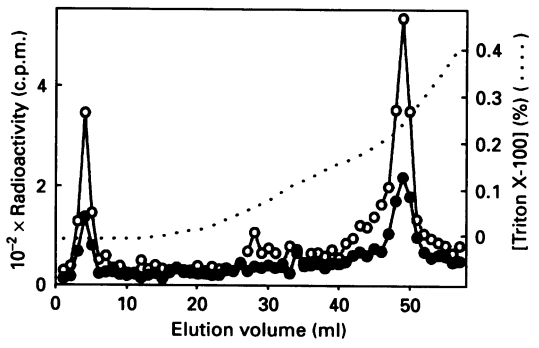


Fig. 2. Octyl-Sepharose CL-4B chromatography in 4 M-guanidinium chloride of UMR 106-01 (BSP) heparan sulphate proteoglycans

Proteoglycans were metabolically labelled as indicated in Fig. 1. ●, ^{35}S; ○, ^{3}H.

Photoaffinity labelling of UMR 106-01 cell membrane with [^{125}I]TID and incorporation into cell-layer-associated heparan sulphate proteoglycan

In order to determine whether some of the cell-associated heparan sulphate proteoglycan was intercalated in the plasma membrane, we employed a highly hydrophobic photoactivatable iodinated precursor ([^{125}I]TID) which has been shown to partition into the lipid bilayer [31] and, when activated, to label specifically those amino acids which comprise the membrane-spanning domain of intercalated proteins. Plasma membranes of UMR 106-01 (BSP) cells that had been incubated overnight in the presence of [^{35}S]sulphate and [^{3}H]leucine were treated with [^{125}I]TID and then solubilized in 8 M-formamide/2% Triton X-100. This extract was applied directly to Q-Sepharose, which was then washed extensively to remove unincorporated [^{125}I]TID and unbound proteins. The bound fraction was step-eluted with 4 M-guanidinium chloride and then chromatographed on Superose 6 eluted in 8 M-formamide/0.2 M-NaCl/50 mM-sodium acetate, pH 6.0, containing 0.5% Triton X-100 (Fig. 3). The ^{35}S profile (Fig. 3*a*, ●) shows predominantly high-molecular-mass material, which contains the intact heparan sulphate proteoglycan and two distinct chondroitin sulphate proteoglycans that are closely associated with the pericellular matrix of these cells [5]. There was very little low-molecular-mass ^{35}S material, indicating the absence of intracellular degradation products as would be expected since they are unlikely to co-purify with the plasma-

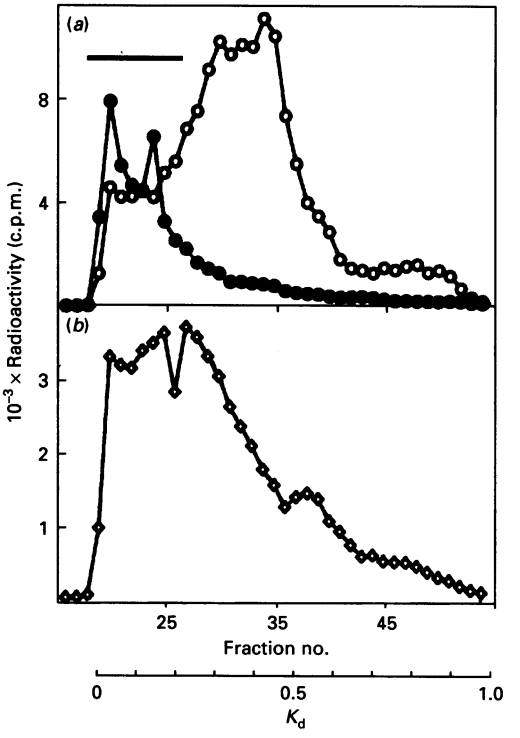


Fig. 3. Superose 6 chromatography of the [^{125}I]TID-labelled plasma-membrane proteoglycan pool

UMR 106-01 (BSP) cells were labelled overnight with [^{35}S]sulphate (●) and [^{3}H]leucine (○), and the plasma-membrane fraction was isolated and labelled with [^{125}I]TID (◇). (*a*) ^{35}S and ^{3}H radioactivity; (*b*) ^{125}I activity. The bar indicates fractions pooled for further analysis.

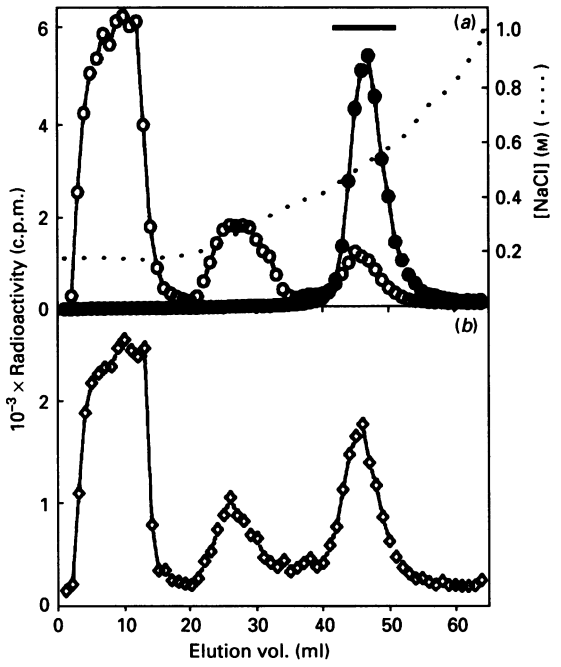


Fig. 4. Q-Sepharose chromatography of [^{125}I]TID-labelled proteoglycans

The proteoglycan pool from Fig. 3 was chromatographed on Q-Sepharose and the ^{35}S and ^{3}H radioactivity (*a*) and ^{125}I activity (*b*) monitored. The bar indicates fractions pooled for further analysis. Symbols are as for Fig. 3.

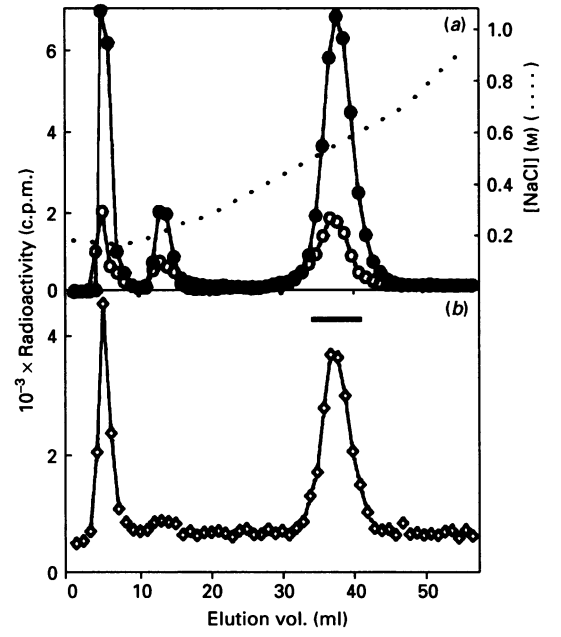


Fig. 5. Q-Sepharose chromatography of the [^{125}I]TID-labelled proteoglycan pool after chondroitinase ABC digestion

(*a*) ^{35}S and ^{3}H radioactivity; (*b*) ^{125}I radioactivity. The bar indicates fractions pooled for further analyses. Symbols are as for Fig. 3.

membrane fraction. Both the [^{3}H]leucine (Fig. 3*a*, ○) and the [^{125}I]TID (Fig. 3*b*) were incorporated into a wide spectrum of species eluted from the Superose 6 column.

The high-molecular-mass ^{35}S-labelled material (Fig. 3*a*, horizontal bar) was pooled and applied to a column of Q-Sepharose followed by extensive washing and subsequent elution of bound material by a NaCl gradient (Fig. 4). A major portion of the ^{3}H (Fig. 4*a*, ○) and ^{125}I (Fig. 4*b*) were unbound, even though they bound to Q-Sepharose in the initial step (see above). This illustrates the difficulties associated with purifying membrane-associated proteoglycans from contaminating proteins [17]. An early eluted peak of ^{3}H and ^{125}I in the salt gradient was not associated with any appreciable ^{35}S activity and probably represents cell membrane glycoproteins. The ^{35}S-labelled proteoglycan pool eluted as a single peak at ~ 0.6 M-NaCl (Fig. 4*a*, ●) and was coincident with a ^{3}H-labelled (Fig. 4*a*, ○) and ^{125}I-labelled (Fig. 4*b*) peak.

The proteoglycan pool (Fig. 4*a*, horizontal bar) was concentrated by a Centricon-10 ultrafiltration device and incubated with chondroitinase ABC. The digest was applied to Q-Sepharose (as described above) and eluted with a continuous NaCl gradient (Fig. 5). The products of the chondroitinase ABC digestion distributed into two pools of ^{35}S activity (Fig. 5*a*, ●): an unbound fraction, which most likely contained chondroitin sulphate disaccharides, and a weakly bound fraction, where the chondroitin sulphate proteoglycan core protein with sulphated chondroitin sulphate-linkage oligosaccharides would be eluted. Approx. 60% of the ^{35}S radioactivity was eluted at ~ 0.6 M-NaCl as intact heparan sulphate proteoglycan, which is resistant to digestion by chondroitinase. The ^{3}H activity was resolved into three peaks; the first unbound peak probably represents contaminating proteins not removed in prior passages through the Q-Sepharose columns; the second peak was eluted early in the gradient and was coincident with the expected position of glycoproteins and core proteins; and the third peak was co-incident with the ^{35}S-labelled heparan sulphate proteoglycan. The ^{125}I-labelled material (Fig. 5*b*) separated into an unbound pool that most likely consisted of contaminating proteins and a major peak co-eluted with the heparan sulphate proteoglycan at ~ 0.6 M-NaCl, strongly suggesting that the heparan sulphate proteoglycan was labelled by the [^{125}I]TID precursor. Very little of the ^{125}I label was associated with the chondroitin sulphate proteoglycan core protein.

The ^{35}S-, ^{3}H- and ^{125}I-labelled heparan sulphate proteoglycan pool (Fig. 5*b*, horizontal bar) was concentrated by a Centricon-10 device and the solvent exchanged into 0.1 M-Tris/0.1 M-acetate, pH 7.3. Aliquots were eluted on Superose 6 in 4 M-guanidinium chloride, with or without prior digestion with heparitinase (Fig. 6). The intact heparan sulphate proteoglycan (Figs. 6*a* and 6*b*) was eluted at a K_d of 0.17 (~ 150 kDa), with all three isotopes co-incident, further supporting the conclusion that the heparan sulphate proteoglycan was labelled by the [^{125}I]TID. Whether the ^{125}I was associated with heparan sulphate chains or the core protein was demonstrated by enzymically removing the heparan sulphate chains to generate a core-protein preparation (Figs. 6*c* and 6*d*). The ^{35}S radioactivity was eluted near the column V_t as heparan sulphate digestion products (Fig. 6*c*, ●), whereas the ^{3}H-labelled core protein was eluted at a K_d of 0.55 (~ 70 kDa), Fig. 6*c* (○), and was co-incident with the ^{125}I activity (Fig. 6*d*). This demonstrates clearly that the [^{125}I]TID specifically labelled the core protein of the heparan sulphate proteoglycan.

Effects of trypsin treatment on membrane-associated heparan sulphate proteoglycan

Confluent cultures of UMR 106-01 (BSP) cells were metabolically labelled with [^{35}S]sulphate and [^{3}H]glucosamine for 20 h, after which the cell layer was washed several times with basal medium. Some cultures were then incubated with trypsin for 30 min, and the heparan sulphate proteoglycans released by this treatment were analysed. Our previous studies [5] have shown that, after trypsin treatment, only low-molecular-mass intracellular degradation products remain associated with the cell layer. The trypsin-released material was incubated with

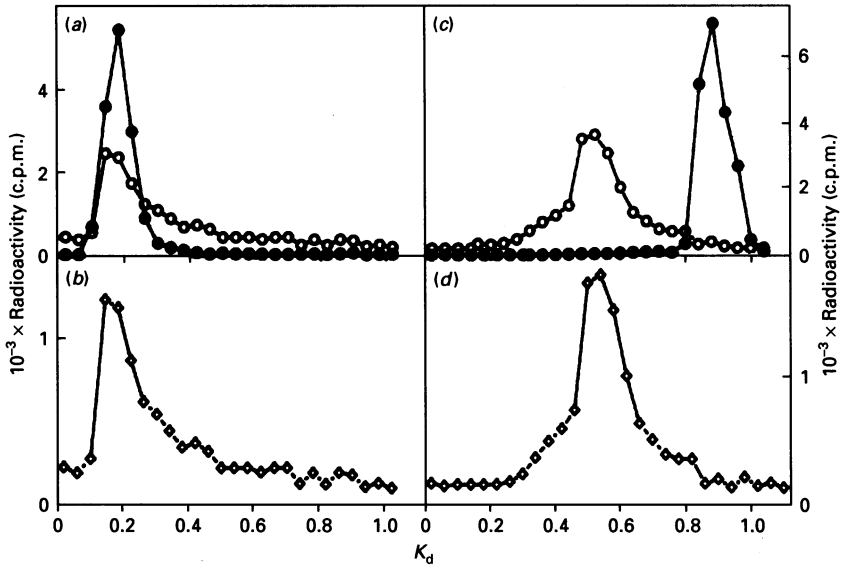


Fig. 6. Superose 6 chromatography in 4 M-guanidinium chloride of [^{125}I]TID-labelled heparan sulphate proteoglycans before and after heparitinase digestion

(*a*) Intact proteoglycan, ^{35}S and ^{3}H radioactivity; (*b*) intact proteoglycan, ^{125}I radioactivity; (*c*) after heparitinase digestion, ^{35}S and ^{3}H radioactivity; (*d*) after heparitinase digestion, ^{125}I radioactivity. Symbols are as for Fig. 3.

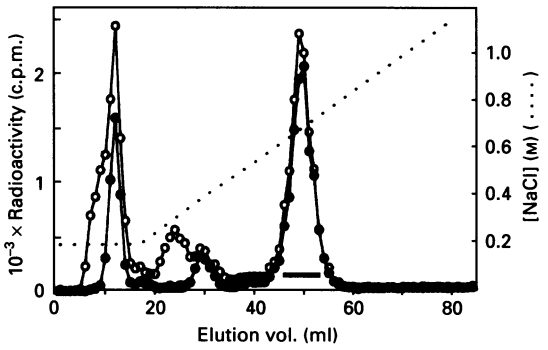


Fig. 7. Purification of trypsin-released heparan sulphate proteoglycan labelled with [^{35}S]sulphate and [^{3}H]glucosamine

Q-Sepharose chromatography was performed after digestion of the proteoglycan pool with chondroitinase ABC. The bar indicates fractions pooled for further analysis in Figs. 8(*a*) and 8(*b*). Symbols are as for Fig. 2.

chondroitinase ABC and then applied to Q-Sepharose (Fig. 7). Bound proteoglycans were eluted with an NaCl gradient and the ^{35}S and ^{3}H radioactivity was monitored. As described above, the chondroitin sulphate disaccharides and core proteins generated by the digestion were eluted in the unbound and early eluting fractions respectively. The heparan sulphate proteoglycan was eluted at ~ 0.6 M-NaCl.

The heparan sulphate pool (Fig. 7, horizontal bar) was then chromatographed on a Superose 6 column, both with and without prior digestion with heparitinase. In the absence of heparitinase (Fig. 8*a*) the trypsin-released heparan sulphate proteoglycan eluted at a K_d of 0.17 (~ 150 kDa), identical with that observed for the heparan sulphate proteoglycan species isolated intact from the cell layer. However, after heparitinase digestion (Fig. 8*b*), both the ^{35}S and ^{3}H radioactivity was co-eluted near the V_t of the column. This is in contrast with a heparan sulphate proteoglycan preparation from a separate experiment isolated directly from the cell layer, in the absence of trypsin, which yielded a ^{3}H-labelled core protein after heparitinase digestion (Fig. 8*c*) at a K_d of 0.55 (~ 70 kDa). These data argue that heparan sulphate chains are clustered in a domain of the core protein separate from that where the smaller oligosaccharides reside. Trypsin treatment yields this peptide domain, substituted with all the heparan sulphate chains, which is eluted on Superose 6 near where the intact heparan sulphate proteoglycan is eluted.

Effects of membrane-anchor-specific phospholipase C digestion

We showed previously [17] that a subpopulation of membrane-associated heparan sulphate proteoglycans in ovarian granulosa cells were intercalated into the plasma membrane through a linkage structure involving phosphatidylinositol. Proteins which share this type of membrane linkage are identified, at least in part, by their susceptibility to phosphatidylinositol-specific phospholipase C (PLC) [32]. We tested the effect of phospholipase C (*B. thuringiensis*) and studied the metabolic clearance of GPI-anchored and non-GPI-anchored cell-surface proteoglycan on cultures of UMR 106-01 (BSP) cells labelled with [^{35}S]sulphate. After a 20 h incubation with [^{35}S]sulphate, cells were washed extensively, chased for 1 h and the chase medium removed to reduce the amounts of ^{35}S-labelled BSP, which is rapidly secreted from the cell layer. Then cultures were divided into three groups. The first group of cultures (zero time) were sequentially treated with phospholipase C and trypsin. Released materials after each enzyme treatment, and the remaining cell layers, were analysed for proteoglycan. The second group of cultures (9 h chase control) were analysed as for the first group after a chase for 9 h in fresh medium. The third group of cultures (9 h chase after PLC) were analysed as for the second group, except after treatment with PLC during the last 30 min of the pre-chase period. This experimental protocol is illustrated in the inset scheme in Table 1. Fractions were analysed for total ^{35}S-labelled macromolecules by Sephadex G-50 chromatography, and for heparan sulphate proteoglycan by chondroitinase ABC digestion followed by Superose 6 chromatography.

The results are summarized in Table 1 and indicate that GPI-anchored heparan sulphate proteoglycans represent about 36% of the cell-surface (i.e. trypsin-accessible) population at zero time.

The GPI-anchored species are catabolized quite rapidly (from

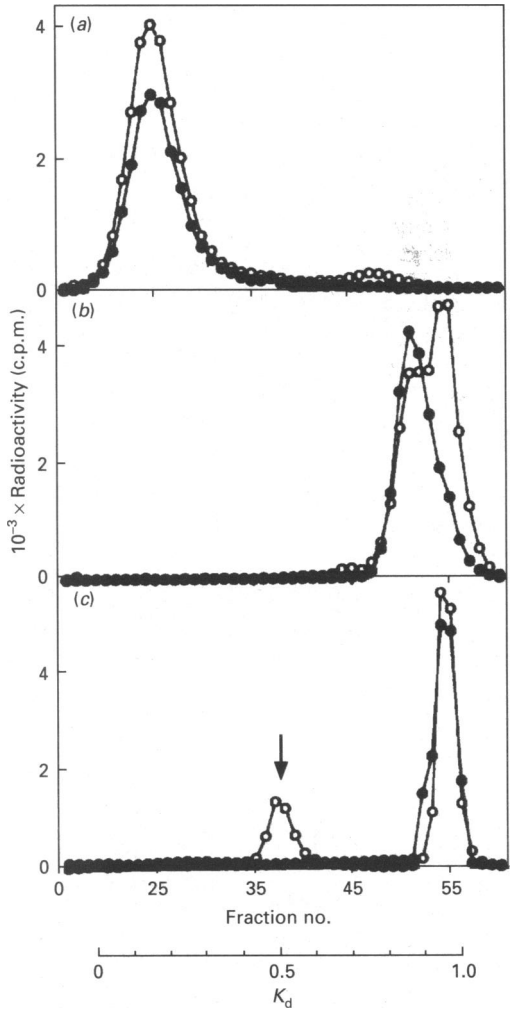
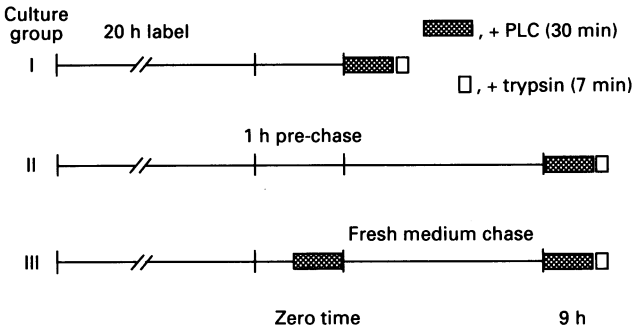


Fig. 8. Superose 6 chromatography of trypsin-released heparan sulphate proteoglycan

(*a*) Before heparitinase digestion; (*b*) after heparitinase digestion; (*c*) heparan sulphate proteoglycan isolated as in (*a*) without prior treatment with trypsin and chromatographed on Superose 6 after heparitinase digestion. The arrow indicates heparan sulphate core protein. Symbols are as for Fig. 2.

Table 1. Distribution of heparan sulphate proteoglycan (HSPG) in UMR 106-01 (BSP) cells

Cells were labelled for 20 h with ^{35}S, washed, and chased for 1 h (pre-chase period) to eliminate BSP from the cell layer. During the final 30 min of the pre-chase period, some cultures were incubated with PLC and the medium collected. Each chase medium, enzyme digest and cell extract was analysed as detailed in the Experimental section. Values represent the means of duplicate determinations, with the range of each set of duplicates indicated. Data shown are from one experiment, with data from a second identical experiment varying by less than 10%. The experimental protocol is shown schematically below:



Culture group...	I (zero time)	II (9 h chase control)	III (9 h chase after PLC)
Cell surface (trypsin-accessible)			
Total*	44.6	18.2	12.5
GPI-anchored	16.0±1.5	3.3±0.4	0.5±0.1
PLC-resistant	28.6±2.6	14.9±1.7	12.0±1.4
Intracellular (trypsin inaccessible)			
Total	55.4±0.5	28.4±2.6	32.4±2.9
Intact HSPG†	14.3	ND‡	4.5
HS chains	41.1	ND	27.9
Release into medium		4.6±0.4	3.6±0.4

* Percentage of total ^{35}S-labelled heparan sulphate proteoglycan at zero time.
† Pooled duplicates analysed by Superose 6 chromatography.
‡ ND, not determined.

16.0 to 3.3% in 9 h), with an average $t_{\frac{1}{2}}$ of about 4 h, compared with the remainder of the cell-surface species (from 28.6 to 14.9%), average $t_{\frac{1}{2}}$ about 9 h. Removal of GPI-anchored species during the pre-chase period did not significantly affect the amount of heparan sulphate proteoglycan secreted into the medium during the 9 h chase period, indicating that the majority (> 90%) of the GPI-anchored species (disappearance of GPI-anchored heparan sulphate proteoglycan from the cell surface was 16.0−3.3 = 12.7%) are not released into the medium but are primarily endocytosed and degraded by lysosomal pathways.

It has been suggested that insulin may alter the rate of turnover of cell-surface heparan sulphate proteoglycans [18], so an experiment similar to that described above was carried out in the presence and absence of insulin (Table 2). It was observed that insulin did not affect the rate of proteoglycan synthesis; however, the rate of secretion over a 2 h chase period was about 12% compared with a control value of about 1%. This enhanced secretion was predominantly accounted for by a decrease in cell-layer-associated PLC-resistant heparan sulphate proteoglycan, indicative of little or no change in the metabolism of the GPI-anchored species.

DISCUSSION

The major heparan sulphate proteoglycan isolated from UMR 106-01 (BSP) cells has a core protein of 70–80 kDa substituted with two to four heparan sulphate chains of ~ 30 kDa each [5]. Trypsin (and papain; R. J. Midura, unpublished work) digestion of the heparan sulphate proteoglycan indicates that the heparan sulphate chains are clustered in a domain separate from the oligosaccharides and render this domain proteinase-resistant. Consistent with possible intercalation in the plasma membrane, this proteoglycan is highly hydrophobic, as assessed by elution from octyl-Sepharose, and is tightly associated with the membrane of the cell, requiring high detergent concentrations for efficient extraction. These characteristics are similar to those ascribed to the major cell-associated heparan sulphate proteoglycans from rat ovarian granulosa cells [6] and a rat parathyroid cell line [7]. We have shown here that the core-protein preparations isolated from these species by heparitinase digestion co-migrate on SDS/PAGE. However, it is important to note that this observation was only possible when preparations were run together on the same gel under identical conditions, owing to the non-ideal migration of heavily glyco-

Table 2. Effect of insulin (10 μg/ml) on the metabolic turnover of heparan sulphate proteoglycan

Values represent the mean of duplicate determinations with the range of each set of duplicates indicated. Data shown are from one experiment, with data from a second, identical, experiment varying by less than 10%. Approx. 25% of the ^{35}S-labelled heparan sulphate at zero time was degraded to free $^{35}SO_4$ during the 2 h chase in both control and treated cultures. Insulin did not stimulate the absolute rate of ^{35}S incorporation. The potency of insulin stock was verified by proliferation/differentiation of 3T3-L1 cells (results not shown).

	Distribution of heparan sulphate proteoglycan after 2 h chase (% of total ^{35}S-labelled heparan sulphate proteoglycan)	
	Control	Insulin-treated
Cell surface (trypsin accessible)		
GPI-anchored	13.4±1.2	10.8±1.0
PLC-resistant	57.7±3.7	50.0±3.0
Intracellular	27.9±0.3	27.4±1.0
Medium	1.1±0.3	11.8±3.9

sylated proteins on SDS/PAGE. We propose, therefore, that the major heparan sulphate proteoglycan associated with the cell membrane of most cell types is represented by an identical, or closely related, species.

Brunner and co-workers [31,33–35] have developed a series of lipid-soluble photoactivatable probes to investigate the membrane-spanning domains of cell-membrane-intercalated proteins. One of these, [^{125}I]TID, has been shown to label in a highly specific manner those parts of intrinsic proteins that are embedded in the lipid bilayer [34]. We investigated the linkage of the heparan sulphate proteoglycan to the cell membrane using this reagent and demonstrated clearly that the majority of these molecules are accessible to the iodinated reagent, and that it is the core protein portion, or a domain closely associated with the core protein (such as a GPI anchor), of the proteoglycan which is specifically labelled. On the basis of this, and previous studies [17,18], it seemed probable that the core protein was anchored by one of two possible mechanisms to the cell membrane: (i) direct insertion of a protein domain into the lipid bilayer; or (ii) covalent linkage to a phosphatidylinositol molecule [32,36]. The latter mechanism was investigated by use of a bacterial phospholipase C enzyme specific for phosphatidylinositol, and was shown to release about 36% of the membrane-associated heparan sulphate proteoglycan. The remaining 64% of cell-membrane-associated heparan sulphate proteoglycan is likely to be directly inserted into the lipid bilayer via a membrane-spanning peptide domain. The two forms of heparan sulphate proteoglycan were further distinguished by their distinct metabolism: the directly intercalated species could be released into the medium compartment, and this release was enhanced in the presence of insulin; the GPI-anchored population was insensitive to insulin and most likely represents a form that is not secreted but is internalized. It is therefore unlikely in this system that insulin stimulates endogenous phospholipases, as has been observed by others [37]. These observations are consistent with those for ovarian granulosa cells [17; M. Yanagishita, unpublished work], where we suggested that the two species may be represented by similar, but not identical, core proteins, on the basis of subtle migration differences on SDS/PAGE. The procedures employed in the present study would not be expected to resolve these two species. David *et al.* (21) proposed that a cDNA clone they have isolated encodes a heparan sulphate proteoglycan which is GPI-anchored to the cell membrane and is a gene product separate from the species directly intercalated via a protein domain. They further concluded that the GPI-anchored species is the precursor of the form in the medium. This is in contrast with the data in the present study, where it is apparent that it is the PLC-resistant population which is preferentially secreted. In any event, the differences in membrane attachment and metabolic fates clearly suggest that these two species perform distinct roles in the biology of UMR 106-01 (BSP) cells.

The relation of the UMR 106-01 (BSP) heparan sulphate proteoglycans, and those of granulosa cells and parathyroid cells, to the proposed syndecan/fibroglycan/glypican gene families [11,12,21,38] remains unclear at present. Elucidation of the different subclasses and their respective modes of attachment to the cell membrane should facilitate the identification and subsequent assignment of function of this ubiquitous class. However, it remains tempting to speculate on the role of these species in osteoblasts, given the observation that heparan sulphate proteoglycans are involved in interactions with both transforming growth factor-β [39] and fibroblast growth factor [40,41] at the cell surface, and these growth factors are likely to be agonists of osteoblast metabolism.

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