

Membrane Anchoring of Heparan Sulfate Proteoglycans by Phosphatidylinositol and Kinetics of Synthesis of Peripheral and Detergent-solubilized Proteoglycans in Schwann Cells

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Abstract. Previous studies have shown that Schwann cells synthesize both peripheral and integral hydrophobic cell surface heparan sulfate proteoglycans (HSPGs). The experiments reported here were undertaken to investigate the mode of attachment of these proteins to the cell surface and their potential interrelationship. The binding of the hydrophobic HSPGs to membranes appears to be via covalently linked phosphatidylinositol based on the observation that incubation of the detergent-solubilized protein with purified phosphatidylinositol-specific phospholipase C significantly reduces the ability of the HSPGs to associate with phospholipid vesicles in a reconstitution assay. The peripherally associated HSPGs were released from the cells by incubation in the presence of heparin (10 mg/ml), 10 mM phytic acid (inositol hexaphosphate), or 2 M NaCl. These treatments also solubilized basement

membrane HSPGs synthesized by the Schwann cells. These data suggest that the peripheral HSPGs are bound to the surface by electrostatic interactions. The peripheral and hydrophobic HSPGs were identical in overall size, net charge, length of glycosaminoglycan chains, and patterns of *N*-sulfation. To determine whether the peripheral HSPGs were derived from the membrane-bound form by cleavage of the membrane anchor, we examined the kinetics of synthesis and degradation of the two forms of HSPGs. The results obtained indicated the existence of two pools of detergent-solubilized HSPG with fast ($t_{1/2} = 6$ h) and slow ($t_{1/2} = 55$ h) turnover kinetics. The data were consistent with a model in which the peripheral HSPGs were derived from the slowly turning over pool of detergent-solubilized HSPGs.

HEPARAN sulfate proteoglycans (HSPGs)¹ are ubiquitous constituents of mammalian cell surfaces (9, 10). Cell surface HSPGs are thought to be involved in a variety of functions, including cell-cell and cell-matrix adhesion (5, 15, 16, 25, 26) and regulation of cell growth (8, 13, 24).

In spite of their potential importance, detailed knowledge of the structure and metabolism of cell surface HSPGs is still lacking. Previous studies have shown that some cells produce both peripheral and integral forms of cell surface HSPGs that are structurally similar (1, 14, 20). The attachment of the integral HSPGs to the membrane apparently involves a small terminal hydrophobic domain, based upon the observation that nearly intact soluble proteoglycan can be released from the surface of several cell types by trypsin treatment (1, 11, 20, 22). Iozzo et al. (12) have shown that binding of a human colon carcinoma HSPG to membranes is mediated by a small (M_r 5,000) trypsin-derived hydrophobic peptide. Recently, Ishihara et al. (13) have reported that small amounts of HSPG can be released from a cultured hepatocyte cell line by incubation with purified phospho-

tidylinositol-specific phospholipase C. This suggests some proteoglycans are bound to the cell surface by covalent linkage with this membrane phospholipid, analogous to the anchoring of the trypanosome variant surface glycoprotein and several mammalian cell surface glycoproteins (17, 18).

Because of structural similarities between the peripheral and integral forms of cell surface HSPG, it has been proposed that the peripheral proteoglycans are derived from the integral HSPGs by cleavage at the cell surface of either the protein core (10) or the phospholipid membrane anchor (13). In the latter case, it was proposed that cleavage by endogenous phospholipase C separates the HSPG from the hydrophobic membrane anchor, leaving inositol phosphate covalently bound to the proteoglycans. This causes the HSPG to be bound to the membrane via a specific receptor for inositol phosphate (13). Evidence for this model comes from the observation that the proteoglycan could be displaced from the surface of hepatocytes by incubation of the cells in media containing inositol phosphate or similar sugar phosphates (13).

We have been investigating the structure and function of proteoglycans synthesized by rat Schwann cells. Previous studies had shown that Schwann cells synthesize both base-

1. *Abbreviation used in this paper:* HSPG, heparan sulfate proteoglycan.

ment membrane and cell surface HSPGs (19). The cell surface HSPGs include peripheral proteoglycans that can be extracted by solutions containing high salt or heparin, and hydrophobic proteoglycans that are extracted only by solutions containing detergent (1). Experiments in which proteoglycan synthesis was blocked by growing Schwann cell-nerve cell cocultures in medium containing 4-methylumbelliferyl- β -D-xyloside indicated that the Schwann cell proteoglycans are not required for attachment of the Schwann cells to axonal membranes or for myelin formation, but are required for de novo assembly of the Schwann cell extracellular matrix (4). Contact with extracellular matrix is essential for normal terminal differentiation of Schwann cells (2, 3, 7).

The studies reported in this paper were undertaken to determine the mechanism of attachment of the Schwann cell hydrophobic HSPG to the cell membrane and the relationship between the integral and peripheral forms of cell surface HSPG. The results obtained indicated that the hydrophobic HSPGs use phosphatidylinositol as their membrane anchor. Investigation of the kinetics of HSPG synthesis indicated the existence of two pools of detergent-solubilized HSPG. The data are consistent with a model in which only one of these pools is the precursor of the peripheral HSPGs.

Materials and Methods

Cell Culture

Schwann cell cultures were prepared from neonatal rat sciatic nerves as described previously (21). Briefly, the nerves were dissociated enzymatically by incubation for 45 min at 37°C in 0.1% collagenase (CLSIII, Worthington Biochemical Corp., Freehold, NJ), 0.25% trypsin in DME. The cells were plated onto plastic tissue culture dishes ($1-2 \times 10^6$ cells per plate) and fed DME containing 10% fetal calf serum (DME-FCS) and 10 μ M cytosine arabinoside (Sigma Chemical Co., St. Louis, MO). After 5 d, the cells were removed from the dishes by brief trypsinization and incubated for 30 min at room temperature in DME-FCS containing a 1:500 dilution of monoclonal anti-Thy1.1 (from conditioned culture medium of hybridoma cell line T11D7e2, obtained from American Type Culture Collection, Rockville, MD) and a 1:8 dilution of rabbit complement (Sigma Chemical Co.). After this treatment, the cells were plated onto poly-L-lysine-coated culture dishes and fed DME-FCS containing 2 μ M forskolin (Sigma Chemical Co.). In most experiments, before radiolabeling, the cells were switched to the serum-free medium N2 (1-4). For the experiments described here, cultures were used between the first and fifth passage. As reported by Porter et al. (21), these cells are functionally competent to form myelin segments under appropriate conditions. Unless otherwise indicated, culture media and reagents were obtained from Gibco Laboratories (Grand Island, NY).

Radiolabeling

Confluent cultures were labeled with [35 S]O₄ (carrier-free sulfuric acid; ICN Radiochemicals, Irvine, CA) in Ham's Nutrient Mixture F12 supplemented with transferrin (100 μ g/ml) and insulin (5 μ g/ml) as described previously (1, 4, 19). At the end of the labeling period, the medium was removed, the cells were rinsed with 0.05 M sodium phosphate, pH 7.5, 0.15 M NaCl, and the radiolabeled proteoglycans were solubilized as described in the figure legends and Table I.

Proteoglycan Analysis

Extracted proteoglycans were subjected to gel-permeation chromatography on 0.75 \times 30-cm columns of TSK-4000SW (Beckman Instruments, Inc., Palo Alto, CA) eluted with 0.1% SDS, 0.1 M Tris-HCl, pH 7.5, at a flow rate of 1 ml/min. Radiolabeled proteoglycan peaks were identified either by collecting fractions of 0.5 ml and measuring the radioactivity in a liquid scintillation counter, or by monitoring radioactivity with an on-line scintillation detector equipped with a liquid flow cell (Beckman Instruments, Inc.).

Glycosaminoglycan analyses were performed as described previously (1,

4, 19). Glycosaminoglycan chains were released by hydrolysis in 0.2 M NaOH for 18 h at room temperature. Degradation of heparan sulfate chains by nitrous acid was performed in 0.18 M acetic acid, 0.25 M sodium nitrite at room temperature for 2 h. Hydrolysis products were separated on a 0.75 \times 30-cm column of TSK-3000SW (Beckman Instruments, Inc.) eluted with 40 mM NaH₂PO₄ at a flow rate of 1 ml/min. Anion exchange chromatography of glycosaminoglycan chains was performed with a 0.75 \times 7.5-cm column of DEAE-5PW (Beckman Instruments, Inc.) eluted with 0.1 M Tris-HCl, pH 7.5, and a linear gradient of 0-1 M NaCl at a flow rate of 1 ml/min.

Vesicle Reconstitution

Association of hydrophobic proteoglycans with reconstituted phospholipid vesicles was performed with HSPGs solubilized by 5% octylglucoside, 1 M NaCl, 0.1 M Tris-HCl, pH 7.5. Aliquots of detergent-solubilized HSPG were mixed with dipalmitoylphosphatidylcholine (3 mg/ml) (Sigma Chemical Co.) and dialyzed at 4°C against 0.1 M Tris-HCl, pH 7.5, 0.2 M NaCl. The vesicles that form during the dialysis were recovered by centrifugation and then resuspended in the appropriate buffer for further analysis.

In some experiments, before addition of lipids and dialysis, aliquots of solubilized HSPGs were digested with enzymes. Trypsin digestion was carried out at room temperature for 30 min at an enzyme concentration of 50 μ g/ml. Carboxypeptidase digestions were done at 37°C for 30 min with 20 U/ml each of carboxypeptidases A and B (Sigma Chemical Co.). Phosphatidylinositol-specific phospholipase C digestion was carried out at 37°C for 30 min with enzyme activities ranging from 0.1 to 10 μ mol of product per minute per milliliter. This enzyme was purified from culture supernatants of *Bacillus thuringiensis* and was generously provided by Dr. Martin G. Low, Columbia University, New York.

Results

Structure of the Membrane Anchor of the Hydrophobic HSPG

We used a vesicle reconstitution assay to assess modifications of the hydrophobic HSPG that might alter or delete the membrane-anchoring region, in an effort to reveal information about its structure. Schwann cell cultures were extracted with 10 mM phytic acid (see below) to remove peripheral HSPGs followed by 5% octylglucoside, 1 M NaCl to solubilize membrane-bound proteoglycans. Aliquots of solubilized proteoglycans were mixed with phospholipids in a solution containing 5% octylglucoside, dialyzed, and then centrifuged to recover the reconstituted vesicles. To assess the degree of vesicle association of the proteoglycans, aliquots of the vesicle pellets and supernatants were dissolved in 0.1% SDS and subjected to gel-permeation chromatography. Typical results of such an experiment are shown in Fig. 1. When octylglucoside extracts were used in reconstitution experiments \sim 70% of the cell surface proteoglycan (Fig. 1 A, retention time 6.9 min) was associated with the vesicles. None of the basement membrane proteoglycan (Fig. 1 B, retention time 5 min) present in the detergent extracts was associated with the vesicles. The vesicle fraction contained an additional radiolabeled peak (retention time 10 min) that was soluble in 70% ethanol and was insensitive to hydrolysis by trypsin, suggesting it was a sulfolipid. In contrast to these results, when solubilized peripheral HSPGs were used in vesicle reconstitution assays, nearly all of the radiolabeled proteoglycan was found in the supernatant (Fig. 1, C and D).

In several experiments, the percentage of octylglucoside-extracted proteoglycan that associated with vesicles ranged between 45 and 90%. The reason for this variability is not known, but it could reflect incomplete extraction of the peripheral proteoglycans. On the other hand, the values ob-

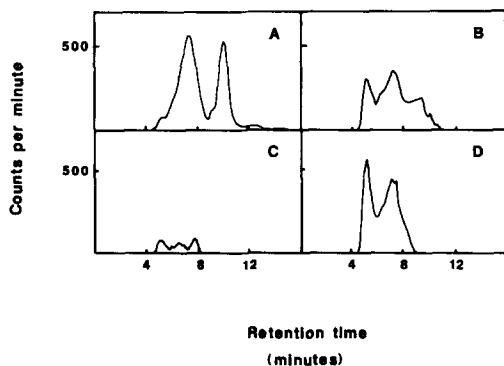


Figure 1. Association of detergent-extracted HSPGs with phospholipid vesicles. Schwann cell cultures were labeled overnight with [^{35}S]O $_4$ and then extracted with 10 mM phytic acid, 0.1 M Tris-HCl, pH 7.5 (15 min on ice), to remove peripheral HSPGs followed by 5% octylglucoside, 1 M NaCl, 0.1 M Tris-HCl, pH 7.5. Aliquots of peripheral or detergent-extracted HSPGs were mixed with phospholipids in buffer containing 5% octylglucoside and the solution was dialyzed to remove the detergent. The resulting vesicles were isolated by centrifugation. Portions of the supernatant fractions or dissolved vesicles were subjected to gel-permeation chromatography on a TSK-4000SW column; radioactivity was monitored with an on-line liquid scintillation detector. Vesicle (A) and supernatant (B) fraction of detergent-extracted HSPGs; vesicle (C) and supernatant (D) fraction of peripheral HSPGs.

tained for replicate measurements made with the same proteoglycan preparation varied by <10%.

Trypsin treatment releases the membrane-bound proteoglycan from Schwann cells in a soluble form that is nearly identical in size to the parent molecule (not shown). Trypsin treatment of detergent-solubilized proteoglycan should, therefore, generate nearly intact proteoglycan lacking the membrane anchor. That this is the case was demonstrated by the observation that vesicle association of trypsin-treated proteoglycan was reduced to only 14% of that of control proteoglycan (Fig. 2).

The data on trypsin-treated proteoglycan suggested the membrane-anchoring portion of the protein is small, since the trypsin-resistant proteoglycan fragment is nearly as large as the parent molecule. A common structural motif of membrane-associated, cell surface proteins that have most of their mass outside the cell is a single membrane-spanning alpha helix near the carboxyl-terminal end of the protein. To test whether this was the case for the cell surface HSPG we digested detergent-solubilized HSPG with a mixture of carboxypeptidases A and B and assayed for the ability of the protein to associate with vesicles. As shown in Fig. 2, carboxypeptidase treatment had no effect on vesicle association.

Evidence has been presented that a hepatocyte HSPG can be released by treatment of the cells with a phosphatidylinositol-specific phospholipase C (13). To determine whether the Schwann cell HSPG uses phosphatidylinositol as its membrane anchor, detergent-solubilized proteoglycans were incubated with purified phosphatidylinositol-specific phospholipase C and then assayed for their ability to associate with reconstituted phospholipid vesicles. Treatment with this enzyme reduced the extent of vesicle association by ~50% (Fig. 2). As shown in Fig. 3, the HSPG lost from the vesicle fraction could be almost entirely accounted for in the supernatant fraction as intact HSPG. These data also demonstrate that phospholipase digestion did not alter the apparent size

of the proteoglycan as determined by gel-permeation HPLC. The extent of reduction in the amount of proteoglycan that associated with vesicles was not changed by increasing the enzyme concentration 100-fold (not shown), indicating the amount of enzyme was not limiting.

Extraction of Peripheral HSPGs

A fraction of HSPGs can be extracted from Schwann cells with solutions containing heparin or high salt concentrations (1). Ishihara et al. (13) have shown that phytic acid (inositol hexaphosphate) is able to extract HSPGs from hepatocyte cultures. Based on this and other data they have suggested that the peripheral proteoglycans are derived from lipid-anchored HSPGs by phospholipase cleavage and are bound to inositol phosphate receptors on the cell surface. We tested solutions containing phytic acid for their ability to extract Schwann cell HSPGs and compared them to solutions containing heparin or 2 M NaCl. These results are summarized in Table I. Phytic acid (10 mM) extracted 46% of ^{35}S -labeled cell surface HSPGs, which was essentially identical to the amounts solubilized by heparin (45%) or 2 M NaCl (52%). That the three solutions were solubilizing the same pool of proteoglycan is indicated by the observation that extraction of the cells with 2 M NaCl after extraction with either phytic acid or heparin solubilized only an additional 17% of the total cell surface proteoglycan.

Results of gel-permeation HPLC of phytic acid-extracted proteoglycans are shown in Fig. 4. Two peaks of radioactivity were observed, corresponding to the basement membrane HSPG (first peak) and cell surface HSPG (second peak). Analysis of heparin- or 2-M NaCl-extracted HSPGs gave identical results (not shown).

Comparison of Peripheral and Hydrophobic HSPG Glycosaminoglycans

The model described above for association of HSPGs with hepatocyte membranes suggests the peripheral cell surface

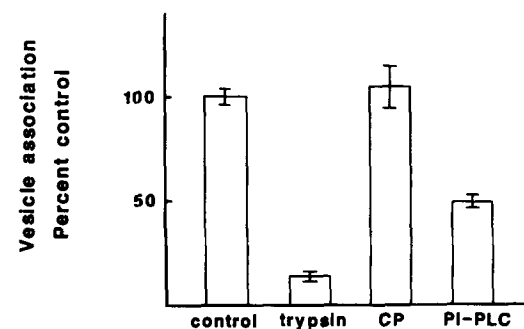


Figure 2. Effect of enzyme treatments on vesicle association of detergent-extracted cell surface HSPGs. Detergent-extractable HSPGs were solubilized with octylglucoside, 1 M NaCl, and used in vesicle reconstitution assays as described in Fig. 1. Before mixing with phospholipids, aliquots of HSPG were incubated with either buffer alone (control), trypsin, carboxypeptidases A and B (CP), or phosphatidylinositol-specific phospholipase C (PI-PLC), as described in Materials and Methods. The amount of radiolabeled cell surface HSPG in vesicle and nonvesicle fractions was determined by means of gel-permeation chromatography as in Fig. 1. The extent of vesicle association after each treatment is shown relative to controls incubated in buffer alone.

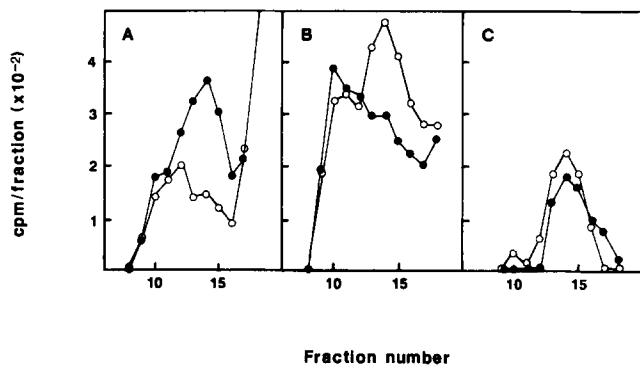


Figure 3. Gel-permeation chromatography of phospholipase-released HSPGs. Detergent-extracted HSPGs were used in phospholipid vesicle reconstitution assays with or without prior incubation with phosphatidylinositol-specific phospholipase C. Aliquots of the vesicle and supernatant fractions were subjected to gel-permeation chromatography on a TSK-4000SW column. Vesicle (A) and supernatant (B) fraction without (●) and with (○) phospholipase incubation; (C) material lost from the vesicles (○) and appearing in the supernatant (●) after enzyme digestion, calculated as the difference between the profiles shown in A and B.

proteoglycans are derived from the membrane-bound proteins by endogenous phospholipase cleavage of the phosphatidylinositol anchor. Analysis of the phytic acid- and detergent-solubilized Schwann cell HSPGs by gel-permeation HPLC gave retention times of 6.8 ± 0.2 min and 6.9 ± 0.2 min (mean \pm SD for seven determinations), respectively, indicating the two HSPGs are very similar or identical in overall size. To compare the glycosaminoglycan structures of the peripheral and hydrophobic cell surface HSPGs, we obtain ^{35}S -labeled glycosaminoglycans by alkaline elimination from the phytic acid-extracted and detergent-extracted HSPGs and compared them in terms of net charge (by elution from an anion exchange column), size (by gel-permeation HPLC), and patterns of *N*-sulfation (by nitrous acid hydrolysis and gel-permeation HPLC). These results are shown in Fig. 5. By all three criteria, the glycosaminoglycan chains obtained from the two HSPG species were indistinguishable.

Time Course of Appearance of Peripheral and Hydrophobic HSPGs

The results presented above indicated the two HSPGs are very similar or identical in structure, which is consistent with the peripheral HSPGs being derived from the membrane-bound form. To determine whether this was the case, we investigated the kinetics of synthesis and degradation of the cell surface HSPGs. Results on the time course of appearance of the peripheral and detergent-extracted HSPGs during continuous labeling with ^{35}S are shown in Fig. 6. Detergent-extractable HSPGs were labeled rapidly and after ~ 6 h began to level off. Half-maximal labeling was observed after ~ 12 h. The phytic acid-extracted HSPGs, after a brief lag, continued to increase in amount until 72 h (the longest time examined). At long labeling times (>24 h) the amount of phytic acid-extracted HSPGs surpassed the amount of detergent-extracted HSPG. Similar results were obtained when 2 M NaCl was used to extract peripheral HSPGs or when Schwann cell-nerve cell cocultures were used (not shown).

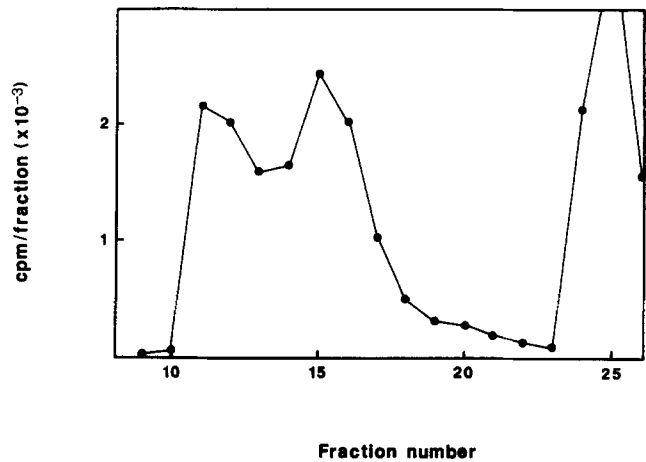


Figure 4. Gel-permeation HPLC of radiolabeled HSPGs released by extraction with phytic acid. Schwann cell cultures were labeled overnight with ^{35}S , the medium was removed, and the cells were incubated with 10 mM phytic acid, 0.1 M Tris-HCl, pH 7.5, for 15 min at 0°C . The radiolabeled HSPGs that were released into this medium were subjected to gel-permeation chromatography on a TSK-4000SW column.

Proteoglycan Turnover Studies

The results presented above are consistent with the possibility that the peripheral HSPGs are derived from the hydrophobic HSPGs. Alternatively, the results are equally consistent with independent synthesis of the peripheral and hydrophobic HSPGs, with the lag in appearance of the peripheral proteoglycans resulting from the time required for their transport to the cell surface from their intracellular site of synthesis. To examine this in more detail, we carried out radiolabeling studies to examine the kinetics of turnover of the proteoglycan. Cultures were labeled with ^{35}S for 24 h and then chased in nonradioactive medium for up to an additional 48 h. As shown in Fig. 7, the kinetics of disappearance of the HSPGs were not representative of simple exponential decay. The curve for the detergent-solubilized fraction appeared to be the sum of two pools of HSPG with short and long half-lives. As shown in Fig. 7, these data could be explained by assuming two pools with half-lives of 6 and 55 h and with 65% of the proteoglycan present in the rapidly turning over pool at time zero.²

The behavior of the peripheral HSPG was even more complex, first increasing and then decreasing during the chase. This pattern is what would be expected if this pool of HSPGs was being added to from another precursor pool. The loss of radioactive HSPG from the rapidly decaying detergent-solubilized pool between 0 and 6 h of chase appeared to be too great to be accounted for by the increase in the peripheral pool during the same period. On the other hand, as shown in Fig. 7, the data were reasonably well fit by a model in

2. The turnover kinetics of detergent-solubilized proteoglycans were modeled by the following equation: $N = N_1 e^{-k_1 t} + N_2 e^{-k_2 t}$. Where N = total amount of detergent-extractable proteoglycan at time t ; N_1 = amount of proteoglycan in pool 1 at time zero; N_2 = amount of proteoglycan in pool 2 at time zero; and k_1 and k_2 are the exponential decay constants for proteoglycans in pools 1 and 2 ($K_x = \ln 2/T_x$, where T_x = half-life of decay of proteoglycan in pool x).

Table 1. Extraction of Cell Surface HSPG

Extractant	Cell surface HSPG extracted
	%
10 mM phytic acid	46
10 mg/ml heparin	45
2 M NaCl	52
2 M NaCl, after phytic acid	17
2 M NaCl, after heparin	17

Schwann cell cultures were labeled for 24 h with [³⁵S]O₄ and then extracted for 15 min on ice with the reagents listed in the Table. All solutions were buffered with 0.1 M Tris-HCl, pH 7.5. The amount of extracted HSPG was determined by subjecting aliquots to gel-permeation HPLC and summing the radioactivity in the second radioactive peak (cell surface HSPG; see Fig. 1). Unextracted HSPGs were solubilized with 2% SDS and quantitated in the same manner. The values are means of determinations made in duplicate cultures. Deviation from the mean was 5% or less.

which it was assumed that all of the slowly turning over detergent-solubilized pool was converted to peripheral proteoglycans, and that once formed, the peripheral HSPGs turned over with a half-life of 65 h.³ Thus, these data indicate that multiple pools of detergent-solubilized HSPGs are present in the Schwann cells. The data are also consistent with the possibility that the peripheral HSPGs are produced by conversion of the slowly turning over pool of detergent-solubilized HSPGs.

Discussion

The results presented here provide evidence that Schwann cells synthesize both peripheral, nonhydrophobic and membrane-associated, hydrophobic HSPGs. The hydrophobic HSPGs appear to use phosphatidylinositol as their membrane anchor. The attachment of the peripheral HSPGs appears to be primarily electrostatic. In their overall size and glycosaminoglycan structure, the two forms of HSPG are indistinguishable. The kinetics of synthesis and turnover of the

3. The turnover kinetics of the peripheral proteoglycans were modeled by the following equation: $N = [N_p - k_2 N_2 / (k_p - k_2)] e^{-k_p t} + [k_2 N_2 / (k_p - k_2)] e^{-k_2 t}$. Where N = the total amount of peripheral proteoglycan at time t ; and k_p = decay constant for the peripheral proteoglycans. k_2 and N_2 are as defined in footnote 2. In using this equation we assumed that the rate of formation of peripheral proteoglycans was equal to the rate of disappearance of membrane proteoglycan from pool 2.

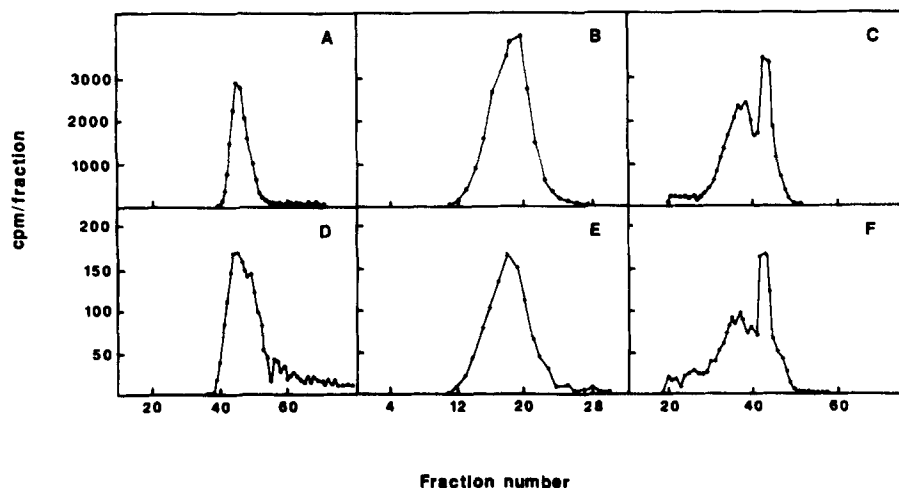


Figure 5. Analysis of glycosaminoglycans of peripheral and hydrophobic HSPGs. Glycosaminoglycan chains were released by alkaline hydrolysis from peripheral (phytic acid-extracted, A-C) and membrane-bound (detergent-extracted, D-F) HSPGs. Aliquots were applied to a column of DEAE-5PW and eluted with a linear gradient of 0-1 M NaCl (A and D); additional aliquots were subjected to gel-permeation chromatography on a TSK-4000 column (B and E) or were digested with nitrous acid and then subjected to gel-permeation chromatography on a TSK-3000 column (C and F).

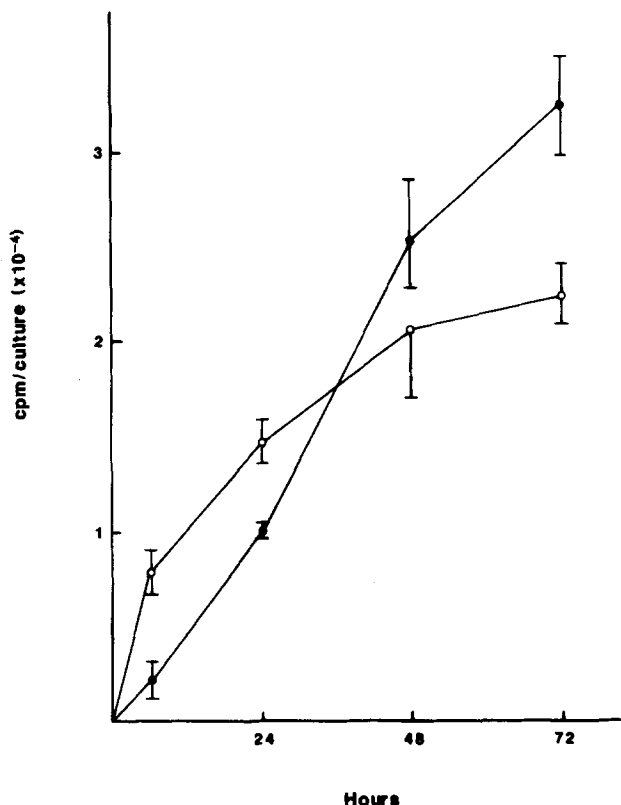


Figure 6. Time course of incorporation of [³⁵S]O₄ into peripheral and detergent-extracted cell surface HSPGs. Schwann cell cultures were labeled with [³⁵S]O₄ for the times indicated. The medium was removed and the cells were extracted with 10 mM phytic acid (●) followed by 2% SDS, 0.1 M Tris-HCl, pH 7.5 (○). Incorporation of isotope into cell surface HSPGs was determined by subjecting the extracts to gel-permeation chromatography on a TSK-4000 column and summing the radioactivity appearing in the cell surface proteoglycan peak. No significant increase in cell number occurred during the labeling period. The values shown and the means ± SD of measurements made on three cultures per time point.

HSPGs were complex. The data are consistent with a model in which two pools of detergent-solubilized HSPG are present, only one of which serves as a precursor for peripheral HSPGs.

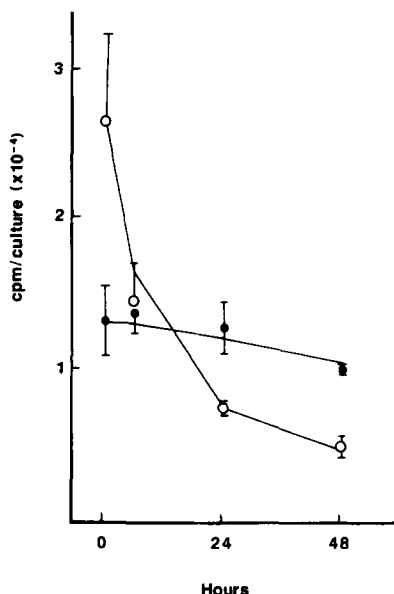


Figure 7. Turnover kinetics of peripheral and detergent-extracted HSPGs. Schwann cell cultures were labeled for 24 h with [³⁵S]O₄, the labeling medium was removed, and the cells were incubated in fresh medium for up to an additional 48 h. At various times after removing the labeling medium, cultures were extracted and radioactivity in cell surface HSPGs was determined as described in the legend to Fig. 6. (●) Phytic acid-extracted HSPGs; (○) detergent-extracted HSPGs. The points indicate the actual measurement values ± SD. The lines were drawn using the values predicted from the model described in the text.

Experiments in which we examined the effects of digestion of the membrane-associated HSPG with phosphatidylinositol-specific phospholipase C on association of the protein with phospholipid vesicles indicated that for a significant fraction of the HSPGs (at least half), association with the membrane is dependent on this phospholipid. This is analogous, presumably, to the situation observed for the trypanosome variant surface glycoprotein and several mammalian cell surface glycoproteins that are bound to the membrane via a covalent linkage between their carboxyl-terminal amino acid and an unusual glycan-phospholipid structure terminating in phosphatidylinositol (17, 18). The reason for the failure of the phospholipase treatment to completely abolish membrane association is not known. This has been observed with other phospholipid-linked proteins and is believed to be due to a structural modification of the glycan-phospholipid structure that renders it insensitive to hydrolysis by the enzyme (17). Whether this is the case or whether there is an additional membrane-anchoring mechanism for the HSPG remains to be determined.

Whether the lipid anchor serves a function in addition to membrane attachment is not known. It has been suggested that it provides a mechanism to rapidly and selectively release proteins from the cell surface by phospholipase cleavage (17). Furthermore, it has been proposed that release of lipid-anchored proteins is regulated by insulin via stimulation of a specific phospholipase C that cleaves the membrane anchors (13, 18). (The serum-free medium used for our labeling experiments contains high levels of insulin.) Analysis of the kinetics of the appearance and disappearance of the

Schwann cell detergent-solubilized and peripheral HSPGs was consistent with this possibility, if it was assumed that only the proteoglycan in the slowly turning over pool of detergent-solubilized HSPGs were converted to peripheral proteoglycans. The validity of this assumption remains to be determined and other more complicated interpretations are possible. For example, the shape of the decay curve for detergent-solubilized HSPGs could result from a single rapidly turning over pool of HSPGs coupled with recycling of the radiolabel. The analysis is complicated by the apparent existence of multiple pools that so far are identified only by their kinetic behavior. Whether these rapidly decaying HSPGs represent entirely different proteoglycans, or perhaps a pool of proteoglycans that are degraded intracellularly before their appearance at the cell surface, is not known.

In the experiments examining the kinetics of synthesis and turnover of cell surface HSPGs, we measured incorporation of [³⁵S]O₄ into peripheral (phytic acid-extractable) and detergent-solubilized HSPGs remaining after phytic acid extraction. It should be pointed out that the latter would include both cell surface membrane HSPGs and intracellular HSPGs. We have observed, however, that the pool of intracellular HSPGs appears to be small, since nearly all of the cell surface HSPG was released by trypsin or phospholipase digestion from cells that had been labeled with [³⁵S]O₄ for 24 h (Carey, D., and R. Stahl, unpublished observations).

Our results also demonstrated that the peripheral cell surface HSPGs synthesized by Schwann cells can be solubilized by phytic acid (inositol hexaphosphate) as well as by heparin and high salt concentration. We do not believe, however, that the association of this HSPG with the cells is via inositol phosphate receptors, as has been suggested for hepatocytes (13). First, the extraction of this protein is not unique to inositol phosphate derivatives, but is a property of highly charged solutes such as heparin or 2 M NaCl. Second, basement membrane HSPG is solubilized by phytic acid as effectively as the cell surface HSPG. Finally, we have observed that solubilized cell surface HSPG binds with high affinity to tissue culture dishes coated with poly-L-lysine, and that phytic acid, heparin, and 2 M NaCl are equally effective at removing the bound HSPG (unpublished observations). The simplest interpretation of these data is that the HSPGs solubilized by these reagents are bound electrostatically to the cell surface and/or the culture substratum, probably via the highly charged heparan sulfate side chains.

These results raise the question of the functional significance of Schwann cells synthesizing both membrane-bound and peripheral forms of what appear to be otherwise identical HSPGs. Our earlier work has shown that inhibition of proteoglycan synthesis does not affect the ability of the Schwann cells to bind to axons or to form myelin in response to basement membrane contact. Under these conditions, however, the Schwann cells are unable to produce the basement membrane that normally surrounds them (4). These observations suggest that the HSPGs do not function as basement membrane receptors, but are involved in basement membrane assembly. The data presented here indicate that a fraction of the cell surface HSPG could be released into the pericellular space to be incorporated into the basement membrane. The remainder would be bound to the cell surface, and could conceivably direct sites of basement membrane assembly by cross-linking laminin and collagen on the

cell surface. The testing of this hypothesis will require the availability of specific antibodies that will allow for the mapping of the distribution of the HSPG in developing and mature nerves or cell cultures.

In our earlier experiments on Schwann cell proteoglycans, we had used primary Schwann cell-nerve cell cocultures derived from rat embryo sensory ganglia (1, 4, 19). The studies reported here were carried out with cultures containing only Schwann cells derived from neonatal rat sciatic nerves. While the results obtained overall were very similar, some differences were observed. In the cocultures grown in serum-free medium, very little of the basement membrane HSPG is associated with the cell layer, whereas in the sciatic nerve cultures, they account for approximately half of the cell-associated HSPGs. This, we believe, is due to electrostatic binding of the basement membrane HSPG to the poly-L-lysine used to coat the culture dishes. In the mixed cultures we used rat tail collagen as a substratum. Another difference between the two preparations is related to the glycosaminoglycan composition of the cell surface HSPG. The proteoglycan isolated from the pure Schwann cell culture contains only heparan sulfate, similar to the HSPG isolated from hepatocytes (10). Our experiments on the Schwann cell plasma membrane HSPG isolated from the embryonic cocultures revealed the presence of both heparan sulfate and chondroitin sulfate in a ratio of 3:1 (1). Cell surface HSPGs with a similar glycosaminoglycan composition have been shown to be synthesized by mouse mammary epithelial cells (6, 23). Whether the chondroitin sulfate is actually part of the Schwann cell HSPG remains to be determined. If it is, this might signify an interesting mechanism for regulating the functional activity of the protein.

We gratefully acknowledge Dr. Martin G. Low, Columbia University, New York, for the generous gift of purified phosphatidylinositol-specific phospholipase C. We thank Phyllis Goldstein for technical assistance. The manuscript was typed by Kathy Knarr.

This work was supported by National Institutes of Health Grant NS 21925.

Received for publication 29 July 1988 and in revised form 20 January 1989.

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