

# Identification of a Lipid-anchored Heparan Sulfate Proteoglycan in Schwann Cells

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**Abstract.** Schwann cells synthesize both hydrophobic and peripheral cell surface heparan sulfate proteoglycans (HSPGs). Previous analysis of the kinetics of radiolabeling suggested the peripheral HSPGs are derived from the membrane-anchored forms (Carey, D., and D. Evans. 1989. *J. Cell Biol.* 108:1891-1897). Peripheral cell surface HSPGs were purified from phytic acid extracts of cultured neonatal rat sciatic nerve Schwann cells by anion exchange, gel filtration, and laminin-affinity chromatography. Approximately 250  $\mu\text{g}$  of HSPG protein was obtained from  $2 \times 10^9$  cells with an estimated recovery of 23% and an overall purification of  $\sim 2000$ -fold. SDS-PAGE analysis indicated the absence of non-HSPG proteins in the purified material. Analysis of heparinase digestion products revealed the presence of at least six core protein species ranging in molecular weight from 57,000 to 185,000. The purified HSPGs were used to produce polyclonal antisera in rabbits. The antisera immunoprecipitated a subpopulation of  $^{35}\text{SO}_4$ -labeled HSPGs that were released from Schwann cells by incubation in medium containing phosphatidylinositol-specific phospholipase C (PI-PLC); smaller amounts of immu-

noprecipitated HSPGs were also present in phytic acid extracts. In the presence of excess unlabeled PI-PLC-released proteins, immunoprecipitation of phytic acid-solubilized HSPGs was inhibited. SDS-PAGE analysis of proteins immunoprecipitated from extracts of [ $^{35}\text{S}$ ]methionine labeled Schwann cells demonstrated that the antisera precipitated an HSPG species that was present in the pool of proteins released by PI-PLC, with smaller amounts present in phytic acid extracts. Nitrous acid degradation of the immunoprecipitated proteins produced a single 67,000- $M_r$  core protein. When used for indirect immunofluorescence labeling, the antisera stained the external surface of cultured Schwann cells. Preincubation of the cultures in medium containing PI-PLC but not phytic acid significantly reduced the cell surface staining. The antisera stained the outer ring of Schwann cell membrane in sections of adult rat sciatic nerve but did not stain myelin or axonal membranes. This localization suggests the HSPG may play a role in binding the Schwann cell plasma membrane to the adjacent basement membrane surrounding the individual axon-Schwann cell units.

**H**EPARAN sulfate proteoglycans (HSPGs)<sup>1</sup> are found on the surfaces of most cells and are believed to play roles in cell-cell or cell-extracellular matrix interactions (10, 13, 23). Molecular cloning and cDNA sequence analysis have provided evidence that some cell surface HSPGs are transmembrane proteins (20, 24). Other cell surface HSPGs, however, appear to be attached to membranes by a glycosylphosphatidylinositol (GPI) anchor (4, 14), similar to the variant surface glycoprotein of trypanosomes and some mammalian cell surface proteins (9, 18, 19). We have shown previously that approximately half of the detergent-solubilized HSPGs made by cultured Schwann cells associate with lipid vesicles via a linkage that is sensitive to hydrolysis by phosphatidylinositol-specific phos-

pholipase C (PI-PLC). The remainder of the hydrophobic HSPGs associate with vesicles by a mechanism that is insensitive to PI-PLC treatment (4). These results suggest that on the Schwann cell surface there are two types of HSPGs that differ in their mechanism of membrane attachment. In addition to these hydrophobic proteoglycans, cultured Schwann cells also possess HSPGs that can be solubilized by highly charged solutes such as heparin or phytic acid (inositolhexaphosphate). Analysis of the kinetics of radiolabeling of the Schwann cell HSPGs with  $^{35}\text{SO}_4$  suggests these peripherally associated HSPGs are derived from the membrane-anchored forms by an as yet unidentified processing mechanism (4).

The function of the Schwann cell HSPGs may be to participate in the interaction of the cells with the extracellular matrix. The myelination and ensheathment of axons by Schwann cells in tissue culture is dependent on Schwann cell contact with basement membrane (5, 6). The Schwann cell HSPGs reversibly bind laminin (Carey, D., and R. Stahl, unpub-

1. *Abbreviations used in this paper:* GPI, glycosylphosphatidylinositol; HSPG, heparan sulfate proteoglycan; PI-PLC, phosphatidylinositol-specific phospholipase C.

lished observations), the major adhesive glycoprotein in basement membranes (7, 11, 26). This binding appears to be mediated by the glycosaminoglycan chains of the HSPGs.

In this paper we describe the purification from Schwann cell cultures of the peripheral cell surface HSPGs. Although several species of core protein were present in these preparations, antisera raised against the purified HSPGs reacted only with a subpopulation of peripheral HSPGs, and with the PI-PLC released form of membrane-anchored HSPG.

## Materials and Methods

### Cell Culture

Schwann cell cultures were prepared from sciatic nerves of 2-d-old rats (Sprague-Dawley strain, Charles River Laboratories, Wilmington, MA) as described previously (4, 22). The cells were maintained on poly-L-lysine-coated tissue culture plates in DME supplemented with 10% FCS, 1% penicillin-streptomycin, and 2  $\mu$ M forskolin. The cells were passaged by trypsinization. In all experiments reported in this paper, we used cells between the first and fifth passage. Primary Schwann cell-nerve cell cocultures were prepared from dissociated dorsal root ganglia of 18–21-d-old rat embryos. The cells were maintained on plastic dishes coated with rat tail collagen in serum-free medium N<sub>2</sub> supplemented with nerve growth factor as described previously (3, 5–7).

### Proteoglycan Purification

Cell surface HSPGs were purified from phytic acid (inositolhexaphosphate) extracts of neonatal rat Schwann cell cultures. Up to 450 75-cm<sup>2</sup> flasks ( $\sim 2 \times 10^9$  cells) of confluent Schwann cells were rinsed three times with ice cold PBS (0.15 M NaCl, 0.05 M sodium phosphate, pH 7.5) and then extracted with 10 mM phytic acid, 0.1 M Tris-HCl, pH 7.5, for 15 min at 0°C. To facilitate identification of the HSPGs some of the cultures were incubated overnight in medium containing <sup>35</sup>SO<sub>4</sub> as described previously (4, 21). The pooled phytic acid extracts were applied to a 2.5  $\times$  15 cm column of DEAE-cellulose equilibrated with 0.1 M Tris-HCl, pH 7.5. This and all subsequent purification steps were carried out in a cold room. After application of the sample the column was washed with 0.1 M Tris-HCl, pH 7.5, until the A<sub>280</sub> of the column effluent was zero. The bound proteins were eluted with a linear gradient of 0–1 M NaCl in 0.1 M Tris-HCl, pH 7.5 (total gradient volume 200 ml). The fractions containing the radiolabeled HSPGs, which eluted at an NaCl concentration of  $\sim 0.5$  M, were pooled and concentrated by dialysis vs. polyethylene glycol (20,000 mol wt). The concentrated sample was applied to a 2.5  $\times$  33 cm column of Sepharose CL-6B equilibrated with 0.1 M Tris-HCl, pH 7.5, and eluted with the same buffer. This step separated the radiolabeled HSPGs into two peaks. The fractions comprising the first peak, which contained basement membrane HSPGs, were pooled and used to prepare a basement membrane HSPG affinity column (see below). The second peak contained the cell surface HSPGs. These fractions were pooled, concentrated as above, dialyzed vs. 0.1 M Tris-HCl, pH 7.5, and then applied to a 1.0  $\times$  6 cm column of laminin-Sepharose that was equilibrated with 0.1 M Tris-HCl, pH 7.5. This column was prepared by covalently coupling purified mouse laminin to CNBr-activated Sepharose (1.0 mg laminin/ml gel) as described previously. The laminin was purified from Matrigel (Collaborative Research, Inc., Bedford, MA), an extract of the Englebreth-Holm-swarm mouse tumor enriched in basement membrane proteins (15), by DEAE-cellulose and gel filtration chromatography, and was pure as judged by SDS gel electrophoresis. After applying the HSPG sample the laminin-Sepharose column was washed with 0.1 M Tris-HCl, pH 7.5, until the A<sub>280</sub> of the effluent was zero. The bound proteins were then eluted with 0.5 M NaCl, 0.1 M Tris-HCl, pH 7.5. Following re-equilibration of the column the proteins in the flow-through were reapplied and the bound proteins were eluted with 0.5 M NaCl, 0.1 M Tris-HCl, pH 7.5. This process was repeated until no additional binding of <sup>35</sup>S-labeled proteins was observed. In earlier HSPG preparations the laminin-Sepharose chromatography step was not used. In these cases, after Sepharose chromatography the HSPGs were subjected to preparative gel permeation HPLC on a 0.75  $\times$  30 cm column of TSK-4000SW eluted with 0.1% SDS, 0.1 M Tris-HCl, pH 7.5 at a flow rate of 1 ml/min.

### Proteoglycan Characterization

Aliquots of purified HSPGs or cell extracts were subjected to analytical gel permeation HPLC on a TSK-4000SW column eluted as described above. 0.5-ml fractions were collected and <sup>35</sup>S radioactivity was determined by liquid scintillation counting. SDS gel electrophoresis was carried out in 7.5% polyacrylamide slab gels prepared as described by Laemmli (16). The gels were silver stained, photographed, and then dried and subjected to autoradiography to localize the radiolabeled HSPGs. HSPGs were digested with heparinase (Seikagaku America, Inc., St. Petersburg, FL) at a concentration of 0.2 U/ml in 10 mM CaCl<sub>2</sub>, 50 mM Tris-HCl, pH 7.0 for 5 h at 37°C. Nitrous acid digestion was carried out according to low pH method of Shively and Conrad (25).

### Antibody Preparation

Polyclonal antisera were produced in rabbits by immunization with purified phytic acid-extracted HSPGs. For each immunization  $\sim 50$   $\mu$ g of HSPG protein was emulsified in Ribi adjuvant system (Ribi ImmunoChem Research Inc., Hamilton, MT) and injected into multiple subcutaneous, intradermal, intramuscular, and intraperitoneal sites. The immunization was repeated 3 wk later and the serum tested for anti-HSPG antibodies 10 d after the second set of injections. The rabbits were boosted as needed using the same injection protocol.

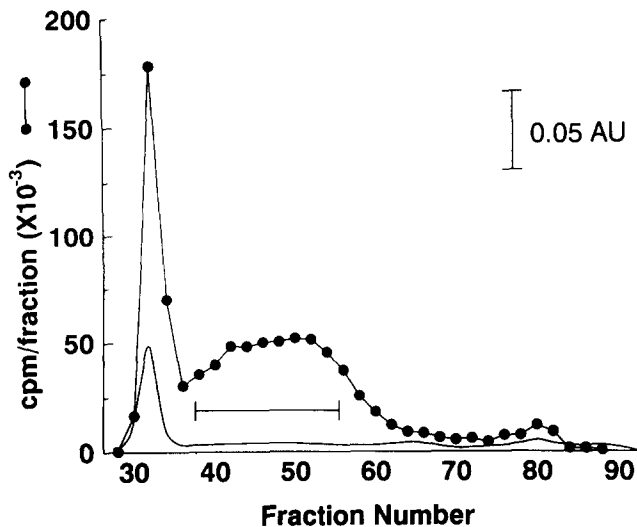
Some antisera showed reactivity towards basement membrane HSPGs in addition to cell surface HSPGs. To remove anti-basement membrane HSPG antibodies the antisera were passed through an affinity column prepared by covalently coupling purified Schwann cell basement membrane HSPGs (obtained from the phytic acid extracts, see above) to CNBr-activated Sepharose. Residual anti-basement membrane HSPG activity was further reduced by incubating the antiserum samples with soluble basement membrane HSPG (1.2  $\mu$ g HSPG protein/ml of antiserum). These treatments effectively eliminated the anti-basement membrane HSPG activity without affecting the ability to immunoprecipitate cell surface HSPGs (not shown). Polyclonal antilaminin antibodies raised against purified mouse tumor laminin have been described previously (5, 6).

### Immunoprecipitation Experiments

Schwann cells were metabolically labeled with <sup>35</sup>SO<sub>4</sub> or [<sup>35</sup>S]methionine as described previously (3, 5). The cells were extracted with 10 mM phytic acid as described above to solubilize the peripheral HSPGs. After this extraction the cells were incubated in N<sub>2</sub> medium containing purified PI-PLC (1 U/ml) for 15–30 min at 37°C, to release the lipid-anchored HSPGs. The enzyme, purified from culture supernatants of *Bacillus thuringiensis*, was generously provided by Dr. Martin G. Low, Columbia University, NY. The membrane-associated HSPGs that remained after these treatments were solubilized with 1% Triton X-100, 0.15 M NaCl, 10 mM Tris-HCl, pH 7.2, 5 mM MgCl<sub>2</sub>, 2 mM EGTA, 0.25 mM DTT. In some cases residual proteins were then solubilized with 2% SDS, 0.1 M Tris-HCl, pH 7.5. Aliquots of the cell extracts were diluted with 1:9 vol of 10  $\times$  immunoprecipitation buffer (5% NP-40, 5% deoxycholate, 1% SDS, 0.5 mM Tris-HCl, pH 7.5), anti-HSPG antiserum was added (1.2  $\mu$ l/10<sup>5</sup> cells), and the samples were incubated overnight at 4°C. A suspension of chromium dioxide particles coated with anti-rabbit IgG antibodies (Magnisort R, DuPont Co., Wilmington, DE) was added to the samples (25  $\mu$ l/ $\mu$ l of anti-HSPG antiserum). After a 5-min incubation at room temperature, the antigen-antibody complexes were sedimented by placing them on a magnetic plate (MagniSort Plate, DuPont Co.). The pellets were washed twice with 1 $\times$  immunoprecipitation buffer and the precipitated proteins were solubilized by resuspending the pellets in buffer containing 2% SDS and heating at 100°C for 5 min. In some experiments the immunoprecipitated proteins were subjected to nitrous acid digestion before solubilization. In this case the washed pellets were resuspended and washed once with water and then resuspended in the nitrous acid reagent. After neutralization the pellets were recovered and the bound proteins solubilized as above.

### Immunofluorescence

Schwann cells to be used for immunofluorescent staining were grown on poly-L-lysine-coated glass slide chambers (Nunc; Lab-Tek Div., Naperville, IL). To stain cell surface antigens the following protocol was used. The cells were chilled on ice, the medium was removed, and the cells were rinsed three times with ice cold PBS and then once with BLOTTO (5% Carnation instant nonfat dry milk, 0.1 M NaCl, 50 mM Tris-HCl, pH 7.4). An-



**Figure 1.** Gel filtration chromatography of Schwann cell peripheral proteoglycans. The proteoglycan peak eluted from a DEAE-cellulose column (see Materials and Methods) was subjected to chromatography on a column of Sepharose CL-6B eluted with 0.1 M Tris-HCl, pH 7.5. Fraction sizes were 2 ml. The solid line is the  $A_{280}$  recorded with an on-line monitor. The bracket indicates the cell surface HSPG fractions that were pooled for further purification.

tisera were diluted in BLOTTO (1:50 dilutions for anti-HSPG antisera) and added to the slide chambers. After a 1-h incubation on ice the antibody solutions were removed, the cells were rinsed three times with cold PBS and then fixed for 30 min with 3% paraformaldehyde in PBS. The cells were rinsed three times with PBS and then incubated for 30 min at room temperature with fluorescein-conjugated affinity-purified goat anti-rabbit IgG (1:20 dilution in BLOTTO). When intracellular antigens were to be stained the cells were fixed as above and permeabilized with 0.05% Triton X-100 in PBS before staining. To stain rat sciatic nerve sections, 2–5- $\mu$ m-thick cryostat sections were fixed with 3% paraformaldehyde, and then stained as described above. The stained cells or tissue sections were examined using a Zeiss Axiovert 35 inverted light microscope equipped for epifluorescence.

## Results

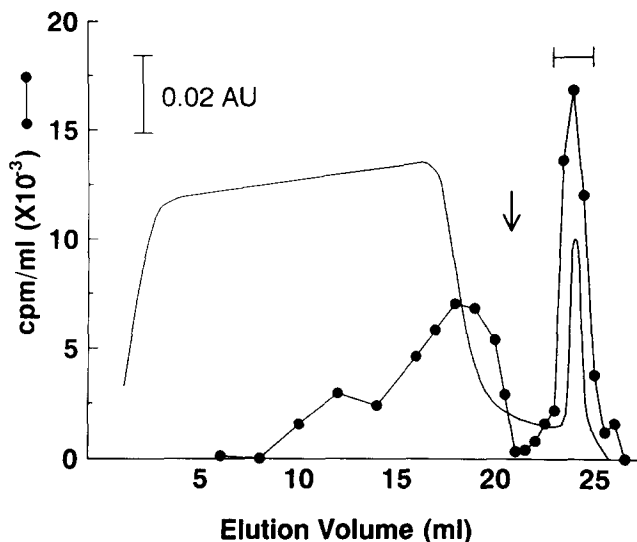
### Purification of Cell Surface HSPGs from Cultured Schwann Cells

To characterize the cell surface HSPGs of Schwann cells we attempted to purify them from Schwann cell cultures in sufficient quantities to allow preparation of specific antibodies and structural analysis. We chose to use the phytic acid-released (peripheral) HSPGs as the starting material since (a) our earlier kinetic studies suggested these were derived from the membrane-anchored HSPGs; (b) the peripheral HSPGs could be solubilized and purified without the use of detergents; and (c) the extraction procedure is relatively selective and yields considerable purification.

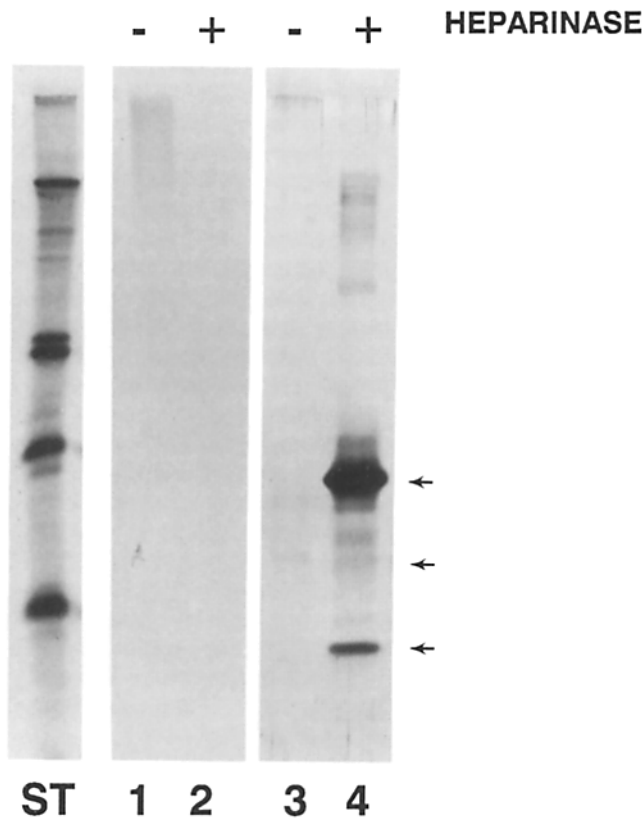
The peripheral HSPGs were solubilized by extraction of confluent cultures of neonatal rat sciatic nerve Schwann cells with 10 mM phytic acid, 0.1 M Tris-HCl, pH 7.5 (4). Before extraction some of the flasks were labeled for 18 h with  $^{35}\text{SO}_4$  to label the HSPGs. Our previous studies have shown that essentially all of the high molecular weight  $^{35}\text{SO}_4$ -labeled molecules synthesized by Schwann cells are HSPGs (21). The pooled extracts were applied to a column of

DEAE-cellulose and eluted with a linear gradient of 0–1 M NaCl. The proteoglycans, which were identified by monitoring  $^{35}\text{SO}_4$  radioactivity, were eluted from the column as a single peak at an NaCl concentration of  $\sim 0.5$  M (not shown). These fractions were pooled, concentrated, and applied to a column of Sepharose CL-6B. As shown in Fig. 1, the radiolabeled HSPGs were eluted from the Sepharose column in two peaks. The sharp peak that was eluted near the column void volume was found to contain basement membrane HSPGs, based on the immunoprecipitation of these HSPGs with two different anti-basement membrane HSPG antibodies (not shown). These fractions were not characterized further. Previous work from our laboratory has shown that the smaller radiolabeled macromolecules are cell surface HSPGs (4, 21). The fractions comprising the broad radiolabeled peak that was eluted after the basement membrane HSPG peak were pooled and applied to a column of laminin-Sepharose. As shown in Fig. 2, all of the radiolabeled molecules were initially bound by the column. A fraction of these were eventually eluted by the large volume of buffer that was needed for the sample loading and column wash. The remainder of the bound radiolabeled molecules were eluted with buffer containing 0.5 M NaCl. When the radiolabeled molecules eluted by the wash buffer were reapplied to the laminin-Sepharose column a profile essentially similar to that shown in Fig. 2 was observed.

When aliquots of the radiolabeled proteins eluted from the laminin-Sepharose column by 0.5 M NaCl were analyzed by SDS gel electrophoresis and silver staining, a broad smear migrating just below the junction of the stacking and separating gels was visible, with no lower molecular weight bands



**Figure 2.** Laminin-affinity chromatography of Schwann cell peripheral HSPGs. The cell surface HSPG peak obtained from the Sepharose CL-6B column (see Fig. 1) was subjected to chromatography on a column of laminin-Sepharose equilibrated with 0.1 M Tris-HCl, pH 7.5. The vertical arrow indicates the beginning of elution of the column with 0.5 M NaCl, 0.1 M Tris-HCl, pH 7.5. The solid line is the  $A_{280}$  recorded with an on-line monitor. The bracket indicates the fractions that were pooled for further characterization and antibody production.



**Figure 3.** SDS-PAGE of purified peripheral HSPGs. Aliquots of the material eluted from the laminin-Sepharose column by 0.5 M NaCl, 0.1 M Tris-HCl, pH 7.5 were subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel without (lanes 1 and 3) or with (lanes 2 and 4) prior incubation with heparinase. Lanes 1 and 2 show the autoradiogram of the dried gel. Lanes 3 and 4 show the gel after silver staining. Arrows indicate protein bands in lane 4 that are present in the heparinase enzyme preparation. *ST* indicates  $^{14}\text{C}$ -labeled molecular weight standards: myosin ( $M_r = 200,000$ ), phosphorylase b ( $M_r = 92,500$ ), BSA ( $M_r = 69,000$ ), ovalbumin ( $M_r = 46,000$ ), and carbonic anhydrase ( $M_r = 30,000$ ).

(Fig. 3). A similar pattern was observed when the  $^{35}\text{SO}_4$ -labeled proteins were visualized by autoradiography (Fig. 3). To demonstrate that the purified material consisted of HSPGs and to identify the core proteins, aliquots were digested with heparinase and then subjected to SDS gel electrophoresis. As shown in Fig. 3, heparinase digestion resulted in the disappearance of the high molecular weight smear on the silver-stained gel as well as on the autoradiogram. The silver-stained gel revealed the presence of at least six new bands in the 57,000–185,000- $M_r$  range (Fig. 3, lane 4). These results indicate that the material eluted from the laminin-Sepharose column consisted of pure HSPGs. The multiple bands visible after heparinase digestion indicate either the presence of multiple core protein species, or the proteolytic degradation of one or a small number of core proteins. The latter could have occurred either during the purification or in situ by the cells.

Table I summarizes the purification of the cell surface HSPGs. The HSPGs were purified nearly 40-fold from the initial phytic acid extract with a recovery of 23%. The yield of purified HSPG from  $2 \times 10^9$  cells was 250  $\mu\text{g}$  of pro-

**Table I.** Purification of Cell Surface HSPGs from Schwann Cell Cultures

	Protein	$^{35}\text{S}$ cpm	cpm/ $\mu\text{g}$ protein	Recovery	Fold purification
	mg	K		%	
Phytic acid extract	39.5	1,950*	49.4	100	1 (52)†
DEAE-cellulose pool	21.0	1,814*	864	93	17 (884)
Sepharose 4B pool	0.684	1,046	1529	54	31 (1612)
Laminin-Sepharose pool	0.250	455	1820	23	37 (1924)

\* Assumes 50% of incorporated radioactivity in cell surface HSPGs, based on gel filtration.

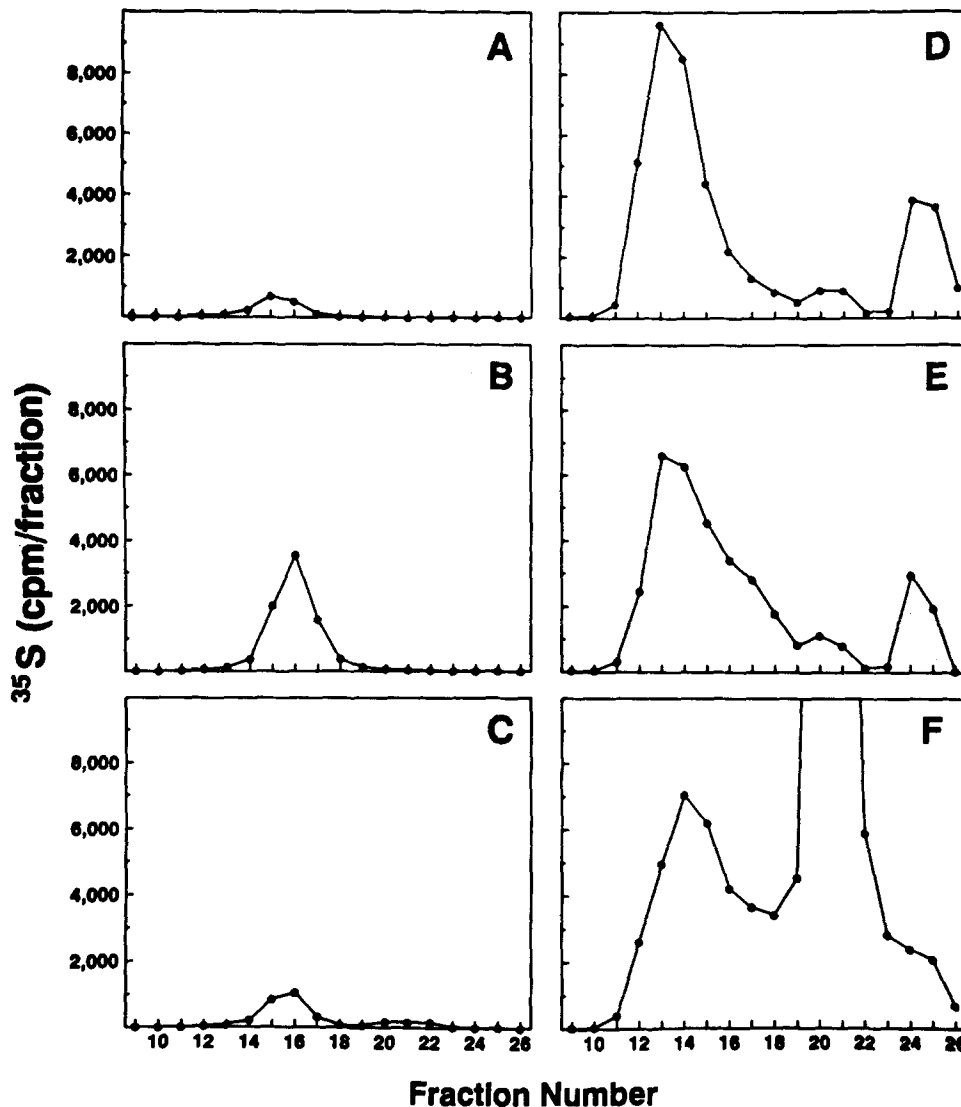
† Numbers in parentheses take into account purification achieved by the extraction.

tein. Since the phytic acid extraction solubilized only 1.9% of the total Schwann cell protein (data not shown) we can calculate an overall purification from the cells of  $\sim 2,000$ -fold.

#### Preparation and Characterization of Anti-HSPG Antibodies

The purified HSPGs were injected into rabbits in order to produce anti-HSPG antibodies. We used immunoprecipitation of  $^{35}\text{SO}_4$ -labeled Schwann cell HSPGs to identify the proteoglycans that were recognized by the antisera. Schwann cell cultures were labeled with  $^{35}\text{SO}_4$  and then treated sequentially with phytic acid (solubilizes peripheral HSPGs), PI-PLC, and detergent. Equal amounts of each extract were reacted with the anti-HSPG antiserum and the precipitates and supernatants were analyzed by gel-permeation HPLC. As shown in Fig. 4, the antiserum precipitated a subpopulation of radiolabeled proteoglycans that were present in largest amount in the PI-PLC-released fraction. The immunoprecipitated proteoglycans were hydrodynamically smaller than the most prominent  $^{35}\text{SO}_4$ -labeled proteoglycans. Both the phytic acid extract and PI-PLC incubation medium contained basement membrane HSPGs that eluted at the column void volume (fraction 13), as well as smaller non-basement membrane HSPGs. The former were identified by their reactivity with anti-basement membrane HSPG antibodies (not shown). The presence of basement membrane HSPGs in the PI-PLC incubation medium was not dependent on PI-PLC, suggesting these were newly synthesized proteoglycans that were secreted during the incubation period. In contrast, the presence of the smaller immunoprecipitated peak was dependent on the presence of PI-PLC in the incubation medium (not shown).

The incomplete immunoprecipitation of the proteoglycans by the anti-HSPG antiserum did not result from insufficient amount of antibody. Re-precipitation of the supernatants or addition of more antiserum to the initial incubations did not result in additional immunoprecipitation. That the immunoprecipitated radiolabeled molecules were HSPGs was confirmed by analysis of degradation products resulting from alkaline elimination and nitrous acid treatment (data not shown).



**Figure 4.** Immunoprecipitation of  $^{35}\text{SO}_4$ -labeled proteoglycans with anti-HSPG antibodies. Schwann cell cultures were incubated overnight in medium containing  $^{35}\text{SO}_4$  and treated as described in Materials and Methods to yield peripheral (A and D), PI-PLC-released (B and E), and detergent-solubilized proteoglycans (C and F). Equal aliquots (by volume) of the extracts were immunoprecipitated with anti-HSPG antiserum prepared by immunizing a rabbit with purified peripheral HSPGs (see Materials and Methods). The immunoprecipitates (A-C) were dissolved in 2% SDS and then subjected to gel permeation HPLC on a TSK-4000SW column equilibrated and eluted with 0.1% SDS, 0.1 M Tris-HCl, pH 7.5. The supernatants from the immunoprecipitation reactions (D-F) were also analyzed. Parallel precipitations with preimmune sera produced profiles only slightly above background (not shown).

The data presented in Fig. 4 were somewhat surprising, since most of the immunoreactive HSPGs were present in a fraction that was not used as the immunogen. To determine whether the immunoprecipitated peripheral (phytic acid-solubilized) HSPGs and the PI-PLC-released HSPGs were being recognized by the same antibodies, we quantitated the immunoprecipitation of HSPGs from radiolabeled phytic acid extracts in the absence or presence of an excess of unlabeled PI-PLC-released proteins. As shown in Fig. 5, the extent of precipitation of the peripheral HSPGs was reduced significantly by a fivefold excess of PI-PLC-released proteins.

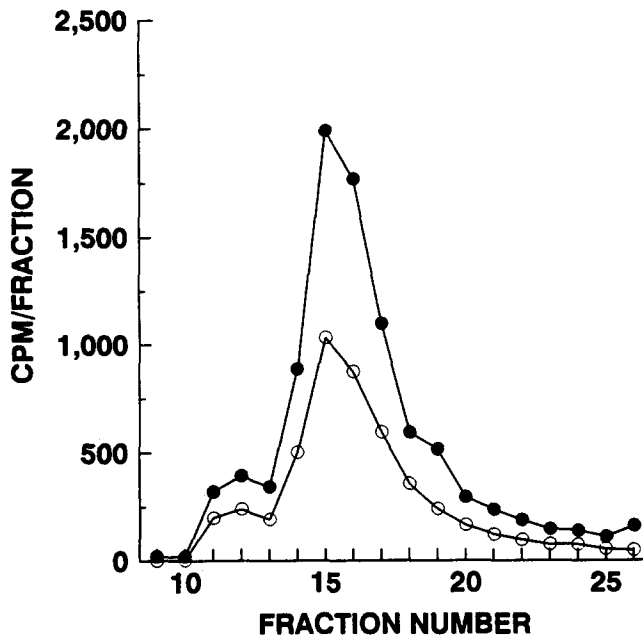
These results indicate that the anti-HSPG antibodies recognize a subpopulation of HSPGs that are released from the cells by PI-PLC treatment. The phytic acid-released (peripheral) pool of HSPGs contains HSPGs that are immunologically related to the PI-PLC-released HSPG.

#### **Identification of the Immunoreactive Core Protein**

The anti-HSPG antisera did not reproducibly stain any polypeptide bands from Schwann cell extracts blotted onto nitrocellulose or Immobilon (not shown). Therefore, to further characterize the anti-HSPG antibodies, extracts of

Schwann cells that had been metabolically labeled with [ $^{35}\text{S}$ ]methionine were immunoprecipitated and analyzed by SDS gel electrophoresis. As shown in Fig. 6 *a*, the antibodies precipitated a PI-PLC-released radiolabeled polypeptide that migrated as a smear in the high molecular weight region of the gel. The apparent molecular weight of the precipitated molecules was smaller than that of the purified phytic acid-solubilized proteoglycans (compare with Fig. 3). This is consistent with the data presented above demonstrating that the anti-HSPG antibodies reacted with a subpopulation of HSPGs that was smaller than the major proteoglycan species (see Fig. 4). Nitrous acid digestion of the immunoprecipitated material resulted in the disappearance of the high molecular weight smear and the appearance of a single broad radiolabeled band migrating at a relative molecular mass of 67,000 (Fig. 6 *a*). All of the immunoprecipitated  $^{35}\text{S}$ -labeled polypeptides remained bound to the antibody-MagniSort complexes after nitrous acid treatment, demonstrating that the antibodies were reacting with the core protein of the HSPGs.

Fig. 6 *b*, lanes 1-3, illustrates the relative distribution of the immunoprecipitated 67,000-*M<sub>r</sub>* band in phytic acid, PI-



**Figure 5.** Immunoprecipitation of peripheral HSPGs.  $^{35}\text{SO}_4$ -labeled phytic acid-extracted HSPGs were immunoprecipitated with anti-HSPG antisera in the absence (●) or presence (○) of a fivefold excess of unlabeled PI-PLC-released proteins. The immunoprecipitates were dissolved in buffer containing 2% SDS and subjected to analytical gel permeation HPLC as in Fig. 4.

PLC, and detergent extracts of Schwann cells (equivalent amounts of each extract were immunoprecipitated and loaded on the gel). This polypeptide was present in the PI-PLC-released fraction (lane 2) but was present in only small amounts in the peripheral (lane 1) and detergent-solubilized (lane 3) fractions. This is consistent with what was observed after immunoprecipitation of  $^{35}\text{SO}_4$ -labeled Schwann cell extracts (see Fig. 4), and supports the conclusion that the anti-HSPG antibodies recognize a PI-PLC-released HSPG.

Fig. 6 *b*, lane 5, shows more clearly the electrophoretic mobility of the immunoprecipitated 67,000-*M*<sub>r</sub> band in the phytic acid extract of Schwann cells. The nitrous acid-treated, phytic acid-extracted (lane 5), and PI-PLC-released (lane 2) polypeptides comigrated during electrophoresis, suggesting that the peripheral and PI-PLC-released immunoreactive HSPGs possessed the same core protein.

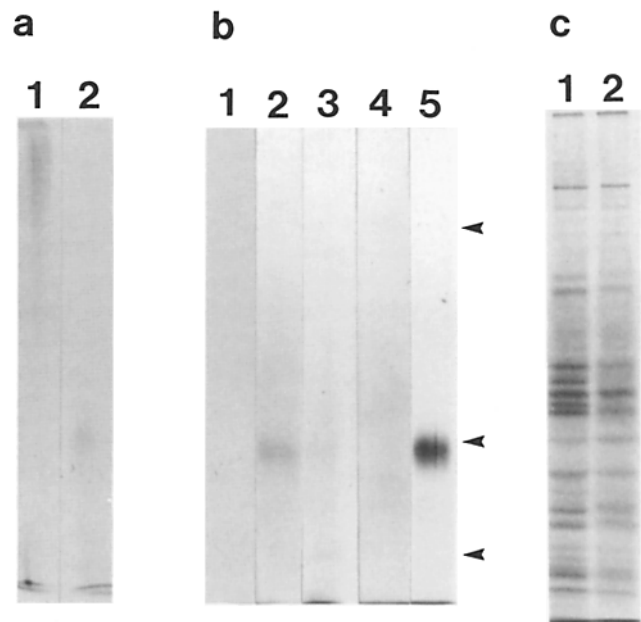
In control experiments nitrous acid treatment did not significantly alter the electrophoretic mobility or autoradiographic intensity of the major [ $^{35}\text{S}$ ]methionine labeled polypeptides in a Schwann cell lysate (Fig. 6 *c*), demonstrating that the nitrous acid treatment does not degrade polypeptide chains.

### Immunolocalization Experiments

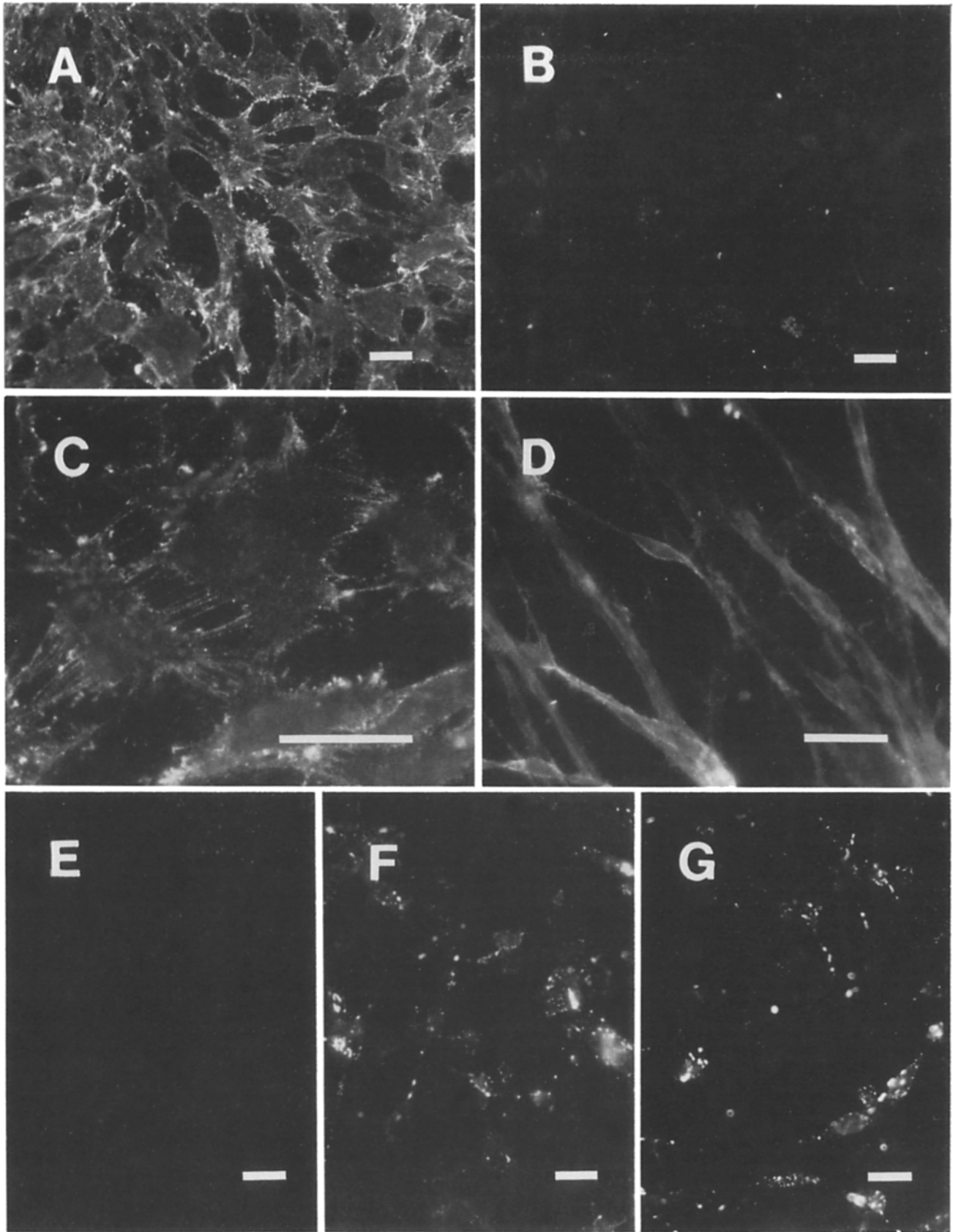
The anti-HSPG antisera were used to stain Schwann cell cultures by the indirect immunofluorescence technique. As shown in Fig. 7, the antisera stained the external surface of the Schwann cells, but the staining was not uniformly distributed. Some regions, especially small spike-like projections visible on many cells, were more intensely stained (Fig. 7 *C*). These projections were rich in actin filaments, based on their intense staining with rhodamine-phalloidin

(not shown). When the Schwann cell cultures were preincubated in medium containing PI-PLC the surface staining was almost totally eliminated (Fig. 7 *B*). In contrast, extraction of the cultures with phytic acid before antibody staining did not visibly alter the pattern or intensity of fluorescent staining (not shown). Thus, the results obtained with immunofluorescence were consistent with the immunoprecipitation data presented above.

The pattern of staining with the anti-HSPG antisera differed from that seen with antilaminin antibodies (Fig. 7 *F*). Consistent with earlier studies (7), we observed punctate surface staining of Schwann cells cultured in the absence of neurons. In contrast to what was observed with anti-HSPG antibodies, the antilaminin staining was not affected by PI-PLC incubation (Fig. 7 *G*). In cocultures of Schwann cells and sensory neurons staining of the surfaces of the Schwann cells with anti-HSPG antisera was observed (Fig. 7 *D*). As with Schwann cell cultures this staining was abolished by preincubation of the cultures in medium containing PI-PLC

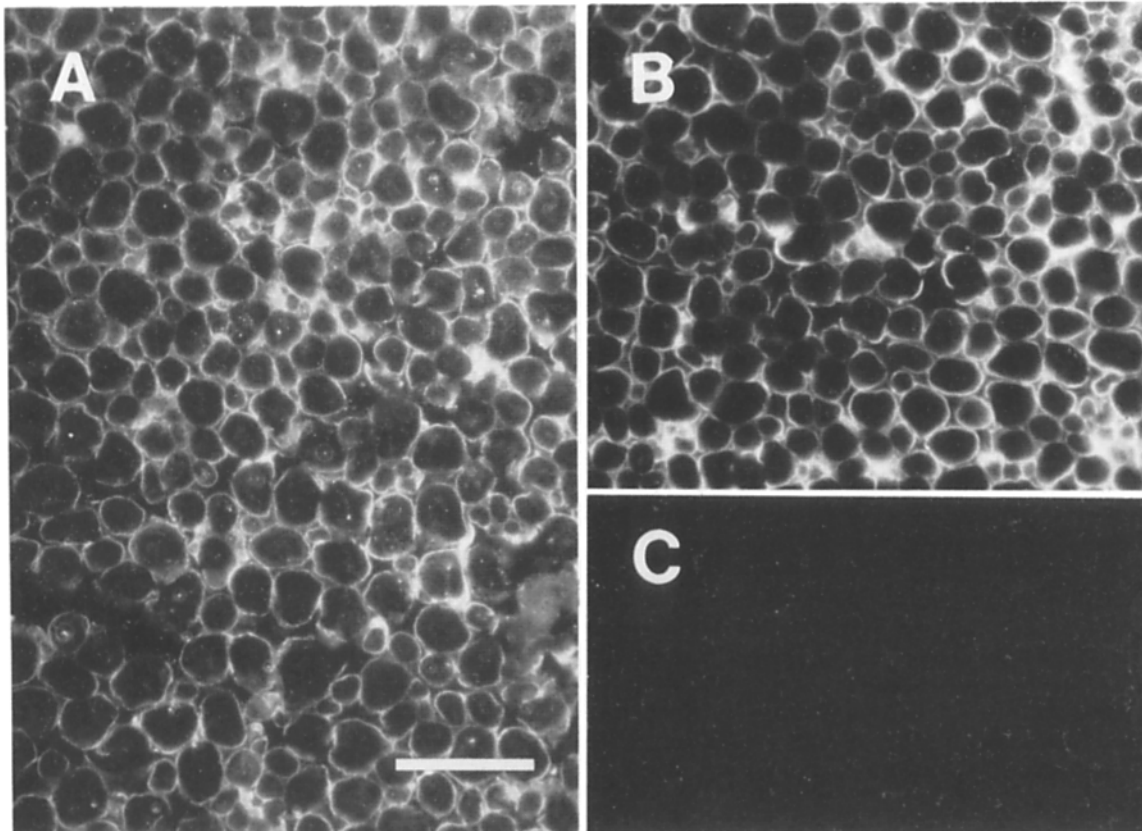


**Figure 6.** Immunoprecipitation of [ $^{35}\text{S}$ ]methionine labeled Schwann cell HSPGs. Schwann cell cultures were incubated for 18 h in medium containing [ $^{35}\text{S}$ ]methionine and then treated to yield peripheral, PI-PLC-released, and detergent-solubilized proteins (see Materials and Methods). (a): PI-PLC-released proteins were immunoprecipitated with anti-HSPG antiserum and subjected to SDS gel electrophoresis without (lane 1) or with (lane 2) nitrous acid treatment before electrophoresis. (b): Equal aliquots (by volume) of phytic acid-solubilized (lane 1), PI-PLC-released (lane 2), and detergent-solubilized (lane 3) proteins were immunoprecipitated with anti-HSPG antiserum; the precipitates were treated with nitrous acid and then subjected to SDS gel electrophoresis. Lane 4 is the precipitate obtained with preimmune serum from the PI-PLC-released fraction. Lane 5 is the anti-HSPG immunoprecipitate of a larger aliquot of phytic acid-solubilized proteins treated with nitrous acid and then subjected to SDS gel electrophoresis. (c): Unfractionated detergent lysates were subjected to SDS gel electrophoresis without (lane 1) or with (lane 2) nitrous acid treatment. The arrows indicate the position of migration of  $^{14}\text{C}$ -molecular weight standards (relative molecular weights of 200,000, 68,000, and 43,000).



**Figure 7.** Cell surface staining of cultured Schwann cells with anti-HSPG antibodies. The cultures were incubated with antiserum before fixation and processed for indirect immunofluorescence (see Materials and Methods). (A) Neonatal sciatic nerve Schwann cells stained with anti-HSPG antibodies; (B) same as (A) except that before staining the cells were incubated in medium containing PI-PLC; (C) high power view of sciatic nerve Schwann cells stained with anti-HSPG antiserum; (D) Schwann cell-nerve cell coculture from embryonic dorsal root ganglia stained with anti-HSPG antiserum; (E) sciatic nerve Schwann cells stained with preimmune serum; (F) sciatic nerve Schwann cells stained with antilaminin antiserum; (G) same as (F) except that before staining the cells were incubated in medium containing PI-PLC. Bars, 50  $\mu$ m.





**Figure 8.** Immunofluorescent staining of sciatic nerve sections. Cryostat sections of adult rat sciatic nerve were stained with anti-HSPG antiserum (*A*), antilaminin antiserum (*B*), or preimmune serum (*C*). Bar, 50  $\mu\text{m}$ .

(not shown). Staining of nerve cells or axons was not observed.

The anti-HSPG antibodies were also used to stain frozen sections of adult rat sciatic nerve. As shown in Fig. 8, the antibodies stained the individual Schwann cell-axon units on the outer Schwann cell surface. In this case the pattern of staining was indistinguishable from what was obtained with antilaminin antibodies (compare Fig. 8 *A* with *B*). Laminin is present in the Schwann cell basement membrane (7). Thus, the anti-HSPG antibodies appeared to be staining molecules present either in the basement membrane or on the Schwann cell plasma membrane adjacent to it. No staining of myelin sheaths, the Schwann cell inner mesaxons, or axons was observed with the anti-HSPG antibodies.

### Discussion

We have described the purification of peripheral cell surface HSPGs from cultures of neonatal rat sciatic nerve Schwann cells. The purified HSPGs appeared to be free of non-HSPG proteins as revealed by SDS-PAGE analysis and silver staining. Analysis of the core proteins comprising this group of HSPGs revealed a surprising degree of complexity, with at least six polypeptides resolved and visualized by SDS gel electrophoresis and silver staining. The source of this complexity is not known, and could indicate multiple core protein species or processing of a smaller group of proteins. A similar degree of core protein complexity of cell surface proteoglycans has been described for a human lung fibroblast

cell line (17). The purified Schwann cell peripheral proteoglycans appeared to contain heparan sulfate as their only glycosaminoglycan. This is in contrast to what we have reported for proteoglycans synthesized by Schwann cells in cocultures with nerve cells (21). This difference may reflect an effect of nerve cells on Schwann cell proteoglycan expression, but this has not been examined directly.

Antisera raised against the purified peripheral HSPGs appeared to be specific for a subpopulation of HSPGs. On immunoblots of Schwann cell proteins the antisera did not stain any polypeptide bands, indicating the absence of immunoreactivity against at least medium and low molecular weight proteins. The reasons for our lack of success in visualizing the HSPGs on immunoblots could include poor transfer to the blotting membrane and the fact that during electrophoresis the HSPGs produce a diffuse smear that would reduce the sensitivity of the staining. Because of this we used immunoprecipitation of extracts of Schwann cells labeled with  $^{35}\text{S}$  or  $^{35}\text{S}$ methionine to identify the immunoreactive proteins. These experiments indicated that the antisera immunoprecipitated a subpopulation of HSPGs that were released by PI-PLC. The antisera immunoprecipitated a single 67,000-*M*, polypeptide that was generated by nitrous acid digestion of PI-PLC-released proteins, and was presumably the core protein of the PI-PLC-released HSPG. This HSPG was also present in the peripheral proteoglycan pool, but at a lower level than in the PI-PLC-released fraction. The 67,000-mol wt immunoreactive polypeptide was not detected in samples that were not nitrous acid treated, indicat-



ing the absence of a large pool of core proteins lacking glycosaminoglycan chains. This HSPG, therefore, is not one of the "part-time" proteoglycans that has been described (1, 10). Interestingly, this is the same molecular weight reported for the core protein of a cell-associated HSPG synthesized by a PC12 cell line. This HSPG is also found in the culture medium of these cells. Its mode of attachment to the cell surface has not been described (12).

Why the antibodies raised against an apparently heterogeneous mixture of purified HSPGs reacted selectively with only a subpopulation of the proteoglycans is not known. This was observed with antisera obtained from 2 of 2 rabbits that were immunized. It appears that the other HSPG core proteins are poor immunogens and did not elicit an immune response in the rabbits.

Our previous analysis of the kinetics of labeling with  $^{35}\text{SO}_4$  of Schwann cell HSPGs suggested the peripheral HSPGs were derived from the membrane-anchored forms (4). The data presented here are consistent with this conclusion, since the PI-PLC-released and a subpopulation of the peripheral HSPGs appear to share a common core protein. This conclusion is based on the immunological cross reactivity of the core proteins and their similar molecular weights as determined by SDS-gel electrophoresis. Final proof of the identity of the core proteins of the peripheral and PI-PLC-released HSPGs must await further structural characterization.

The data also suggest that the PI-PLC-released HSPG possesses a unique core protein that is distinct from those of the PI-PLC-insensitive detergent-solubilized HSPGs. An alternative possibility is that the PI-PLC-released and -insensitive HSPGs utilize a variant form of the same core protein and that the antigenic sites are either absent or masked in the latter form. The likelihood of this, however, is reduced by our use of polyclonal antibodies. Membrane anchoring of the same cell surface protein by both polypeptide and lipid anchors has been demonstrated for the cell adhesion molecule N-CAM (8). We have not yet identified the core protein(s) responsible for membrane anchoring of the PI-PLC-resistant HSPGs. Recent cDNA cloning and sequence analysis has demonstrated the presence in Schwann cells of a polypeptide that is related to the syndecan core protein and contains potential transmembrane and stop-transfer sequences (Evans, D., and D. Carey, unpublished observations). Additional immunological and sequence analysis will be needed to clarify the relationship of these HSPG classes.

Our data indicating that the 67,000-mol wt core protein is released from cells by incubation in medium containing PI-PLC suggests this protein is anchored to the membrane by a GPI moiety, although direct evidence for this is still lacking. This finding could also be explained by the existence of a GPI-anchored protein that selectively binds this HSPG to the surface by a noncovalent association that does not involve electrostatic binding via the heparan sulfate chains. In this case, release of the HSPG from the surface, either in situ by the cells or by exogenous PI-PLC, could be achieved by cleavage of the membrane anchor of the binding protein. It would be expected, however, that the binding protein would remain bound to the HSPG during the purification, since the HSPGs were solubilized by 10 mM phytic acid, which does not release PI-PLC-releasable HSPG, and protein denaturants were not used during the purification. Because SDS gel

electrophoresis analysis did not indicate the presence of non-HSPG proteins in the purified HSPG preparation we favor the simpler explanation that the HSPG itself contains the GPI anchor. Additional experiments will be required, however, to demonstrate this directly.

The function of GPI anchors of cell surface proteins is not known with certainty. They have been suggested to provide a mechanism to rapidly and selectively release cell surface proteins, either to be used at a distance from the cell or to alter the protein composition of the cell surface (9, 18, 19). Data from preliminary experiments suggests this may occur for the PI-PLC-released HSPG (Stahl, R., and D. Carey, unpublished observations). At the present time it is not known whether release of this HSPG involves cleavage of the proposed GPI anchor or a proteolytic cleavage of the core protein near the site of the membrane anchor.

Experiments with hybrid proteins expressed in polarized epithelial cells have indicated that an additional function of GPI anchors may be to provide a signal to direct protein sorting to the apical cell surface (2). Fully differentiated Schwann cells are spatially and functionally polarized, with regions of the Schwann cell membrane specialized to interact with axonal membranes, extracellular matrix, or membrane leaflets of the myelin sheaths. The molecular mechanisms that direct proteins to the proper membrane compartments in Schwann cells are not known. Our data indicate that the PI-PLC-released HSPG is restricted to the region of Schwann cell membrane that is in contact with the basement membrane that surrounds the Schwann cell-axon units.

At the level of resolution provided by light microscopy it is not possible to determine whether the staining observed in sciatic nerve sections corresponded to proteins associated with the plasma membrane of the Schwann cell, the basement membrane, or both. Other experiments carried out in our laboratory have shown that the PI-PLC-released HSPG binds to laminin (our unpublished observations). The localization of the HSPG in sciatic nerve is consistent with its playing a role in the adhesion between the Schwann cell membrane and the basement membrane. These HSPGs, however, are probably not the primary laminin receptors for Schwann cells. Evidence for this comes from our data showing a lack of spatial overlap between the HSPG and laminin on cultured Schwann cells and the observation that PI-PLC digestion did not alter the pattern or intensity of antilaminin staining. Additional information concerning the precise role played by the lipid-anchored HSPG in this process awaits further experimentation.

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