Synthesis by Schwann Cells of Basal Lamina and Membrane-associated Heparan Sulfate Proteoglycans

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ABSTRACT Primary cultures that contain only Schwann cells and sensory nerve cells synthesize basal lamina. The assembly of this basal lamina appears to be essential for normal Schwann cell development. In this study, we demonstrate that Schwann cells synthesize two major heparan sulfate-containing proteoglycans. Both proteoglycans band in dissociative CsCl gradients at densities <1.4 g/ml, and therefore, presumably, have relatively low carbohydrate-to-protein ratios. The larger of these proteoglycans elutes from Sepharose CL-4B in 4 M guanidine hydrochloride (GuHCl) at a K_{av} of 0.21 and contains heparan sulfate and chondroitin sulfate chains of M_r 21,000 in a ratio of ~3:1. This proteoglycan is extracted from cultures by 4 M GuHCl but not Triton X-100 and accumulates only when Schwann cells are actively synthesizing basal lamina. The smaller proteoglycan elutes from Sepharose CL-4B at a K_{av} of 0.44 and contains heparan sulfate and chondroitin sulfate chains of M_r 18,000 in a ratio of ~4:1. This proteoglycan is extracted by 4 M GuHCl or by Triton X-100. The accumulation of this proteoglycan is independent of basal lamina production.

The extracellular matrix (ECM)[1] profoundly affects the behavior and development of many kinds of cells. Unfortunately, however, the detailed mechanisms underlying these effects are usually not known (reviewed in references 1–4). In recent years, significant advances have been made in our understanding of the biochemical nature of the ECM, highlighted by the isolation and characterization of several of its major macromolecular components. Most extracellular matrices contain three classes of macromolecules: collagens, of which there are at least five genetically distinct types; glycoproteins (such as fibronectin and laminin); and proteoglycans (1). Each specific ECM component possesses a distinctive tissue distribution and presumably, a distinct function. Of particular interest from the latter group are heparan sulfate–containing proteoglycans that are present in basal laminae (5–9) as well as plasma membranes (10–12) of certain cells.

Primary cell cultures of embryonic rat Schwann cells and sensory nerve cells provide a useful system for studying the biosynthesis and biological effects of ECM. In these cultures, a basal lamina is formed that covers the outer surface of the Schwann cells and morphologically resembles the Schwann cell basal lamina produced in vivo (13, 14). Biochemical and immunochemical experiments have shown that Schwann cells are the source of the collagens and glycoproteins (primarily laminin) present in the basal lamina (13, 15–17). Basal lamina formation, however, proceeds only in the presence of nerve cells (14). Recent experiments indicate that nerve cells are required for maintaining high levels of production by Schwann cells of type IV collagen, a major constituent of the basal lamina (16). Treatments that prevent basal lamina formation in culture block the later stages of Schwann cell differentiation, namely myelination and unmyelinated ensheathment of nerve fibers (18, 19), suggesting that the basal lamina carries out an important function in peripheral nerve development. The mechanism of this effect of basal lamina on Schwann cells is not understood.

Most of the work to date on the Schwann cell ECM has been either ultrastructural or has concentrated on the collagen and glycoprotein constituents of the matrix. The accumula-

[1] *Abbreviations used in this paper:* ECM, extracellular matrix; GuHCl, guanidine hydrochloride; N2FA, N2 medium supplemented with fetuin and ascorbic acid; PG1 and PG2, proteoglycan fractions.

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tion of evidence indicating that proteoglycans play important roles in ECM (2, 20–23) has prompted us to examine their presence in Schwann cell–nerve cell cultures. Here we report that Schwann cells synthesize two major low-density heparan sulfate proteoglycans that differ in size and apparent distribution.

MATERIALS AND METHODS

Cultures: Primary cultures of Schwann cells and nerve cells were prepared from dorsal root ganglia of embryonic rats (Sprague-Dawley strain, from Dominion Laboratories, Dublin, VA) as described earlier (13, 15, 19, 24). Both explant and dissociated cell cultures were used in the studies reported here and gave similar results.

Radiolabeling: Cultures were labeled with $^{35}SO_4$ (carrier-free sulfuric acid, obtained either from New England Nuclear, Boston, MA, or ICN Pharmaceuticals, Inc., Irvine, CA) by an incubation in Ham's F-12 medium containing 200 μCi/ml $^{35}SO_4$ with or without the additives indicated in Results for up to 48 h. In some experiments, the labeling medium also contained 50 μCi/ml 6-[^{3}H]glucosamine (30 Ci/mmol, ICN Pharmaceuticals, Inc.). At the end of the labeling period, the medium was removed, the cultures were rinsed once with 0.05 M Na-phosphate, 0.15 M NaCl, pH 7.5 containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 4 mM benzamidine, 10 mM EDTA), and the medium and rinse were pooled. The cultures were rinsed twice more with the same buffer. Cultures were extracted overnight at 4°C with 4 M guanidine hydrochloride (GuHCl), 0.05 M Na-acetate, pH 5.0, 0.05% Triton X-150 containing protease inhibitors (0.5 ml/culture). In some experiments, cultures were extracted with 1% Triton X-100, 0.05 M Tris-HCl, pH 7.4 plus protease inhibitors for 1 h at 4°C before GuHCl extraction. To quantitate extraction of $^{35}SO_4$-labeled macromolecules by 4 M GuHCl, the remaining cellular material was dissolved in 0.5 N NaOH. 4 M GuHCl extracted ~75% of the cell-associated macromolecular $^{35}SO_4$.

Proteoglycan Analysis: Radiolabeled proteoglycans were separated from unincorporated isotope by chromatography on a 1 × 30-cm column of Sephadex G-50 with 4 M GuHCl buffer as the eluting buffer. The radioactive material eluting in the void volume was pooled. The radiolabeled proteoglycans were concentrated by precipitation with 1.3% potassium acetate/95% ethanol (−20°C, overnight) and redissolved in 4 M GuHCl buffer or another appropriate solvent. This procedure also eliminated $^{35}SO_4$-labeled organic-soluble material (presumably sulpholipids) from the proteoglycan fractions.

Equilibrium density gradient centrifugation was performed on some proteoglycan samples. Solid CsCl was added to proteoglycans in 4 M GuHCl buffer to a final density of 1.45 g/ml. Centrifugation was done for 48 h in a Beckman SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 35,000 rpm at 10°C. Gradients were fractionated by piercing tubes from the bottom and collecting 0.5-ml fractions.

Proteoglycans were separated preparatively on 1 × 100-cm columns of Sepharose CL-4B equilibrated and eluted with 4 M GuHCl buffer at a flow rate of 6 ml/h. Glycosaminoglycans were liberated from proteoglycan core proteins by hydrolysis in 0.05 M NaOH, 1 M $NaBH_4$ at 45°C for 16 h (25). Sizes of glycosaminoglycans were determined by chromatographing them on a 1 × 100-cm column of Sepharose CL-4B eluted with 4 M GuHCl buffer and calibrated with chondroitin sulfate chains isolated and characterized as described by Wasteson (26). Glycosaminoglycans were treated separately with nitrous acid (27) and chondroitinase ABC (25). Degradation of glycosaminoglycan was determined by chromatography on Sephadex G-50.

Monoclonal Antibody: Details concerning the isolation and initial characterization of monoclonal antibodies that recognize Schwann cell antigens will be presented elsewhere.[2] Briefly, mice were injected with a homogenate of 1-mo-old primary Schwann cell–nerve cell cultures and boosted with a homogenate of rat postnatal day 6 sciatic nerve. Spleen cells from these mice were fused with mouse myeloma cells, and culture media of hybrid clones were screened for antibodies that bound to primary cultures of embryonic rat Schwann cells and nerve cells. The antibody produced by clone B3, used in this study, binds to an antigen present in cultures grown in medium containing serum and chick embryo extract but not in N2 medium. By indirect immunofluorescence, the antibody stains adult rat sciatic nerve sections in a pattern identical to that of basal lamina components such as laminin or collagen type IV. In immunoblot or immunoprecipitation reactions, the antibody does not bind to laminin, fibronectin, collagen, or other known matrix proteins.

Immunoprecipitation: Proteoglycans, extracted from $^{35}SO_4$-labeled cultures with 4 M GuHCl, were dialyzed overnight at 4°C vs 0.05 M Na-phosphate, 0.15 M NaCl, pH 7.5. Ascites fluid containing the mouse monoclonal antibody B3 was added (5 μl/ml of proteoglycan solution) and incubated at 4°C for 3 h. Antigen-antibody complexes were precipitated by the addition of Pansorbin cells (Calbiochem-Behring Corp., La Jolla, CA) (250 μl of a 10% suspension per milliliter of proteoglycan solution). The resulting pellets and supernatants were diluted with 4 M GuHCl buffer and chromatographed on Sepharose CL-4B as described above.

Immunofluorescence: Schwann cell–nerve cell cultures were rinsed with Leibovitz L15 medium and incubated for 1 h at 4°C with B3 antibody (1:200 dilution of ascites fluid) in Leibovitz L15 medium containing 10% heat-inactivated horse serum. The cultures were rinsed with L15-10% horse serum, fixed in 3% paraformaldehyde at room temperature for 30 min and then incubated with affinity purified fluorescein-conjugated goat anti-rabbit IgG (1:20 dilution) for 30 min. After the cultures were rinsed, they were mounted on glass slides and viewed under a Nikon diapot inverted microscope (Nikon Inc., Garden City, NY) equipped for epifluorescence.

RESULTS

Selective Accumulation of a Sulfated Macromolecule during Basal Lamina Formation

Schwann cell–nerve cell cultures can be grown under conditions in which axonal growth and Schwann cell proliferation occur normally, but basal lamina formation, and ensheathment and myelination of axons by Schwann cells do not occur (15, 19). Basal lamina formation can be initiated by adding certain components to the culture medium, which also results in ensheathment and myelination of axons (15, 19; Carey, D., and M. Todd, manuscript submitted for publication). We were interested in identifying proteoglycans that are present constitutively as well as proteoglycans that accumulate only during basal lamina formation. Cultures were grown in the serum-free defined medium N2, in which no ultrastructurally detectable basal lamina is formed (19). After 4 wk in this medium, groups of cultures were switched to N2 medium supplemented with fetuin (1 mg/ml) plus ascorbic acid (50 μg/ml) (N2FA medium) to initiate basal lamina formation (Carey, D., and M. Todd, manuscript submitted for publication). The remaining cultures were kept in N2 medium. After 2 d, both groups of cultures were labeled with $^{35}SO_4$ as described in Materials and Methods. Table I shows the amounts of $^{35}SO_4$-labeled macromolecules in the culture media and 4 M GuHCl extracts of these cells. In the N2 cultures, most of the $^{35}SO_4$-labeled molecules were in the culture medium, whereas in N2FA cultures, a greater portion of the labeled molecules were in the 4 M GuHCl extract. The total amount of $^{35}SO_4$-labeled macromolecules (medium plus cell-associated) in N2FA cultures was approximately twice of that in N2 cultures. This difference was largely because N2FA cultures, which were actively forming basal lamina, accumulated greater than three times more GuHCl-extractable $^{35}SO_4$-labeled macromolecules than did N2 cultures. In contrast, the amounts of $^{35}SO_4$-labeled macromolecules in the culture media in the two types of cultures were nearly the same.

TABLE I. *Effect of Culture Conditions on Accumulation of $^{35}SO_4$-labeled Macromolecules in Schwann Cell–Nerve Cell Cultures*

Experiment	Compartment	N2	N2FA	N2FA/N2
		cpm/culture		
1	Culture medium	11,250	13,000	1.2
	Guanidine extract	5,000	16,300	3.3
2	Culture medium	9,080	11,130	1.3
	Guanidine extract	3,132	10,130	3.2

[2] Cornbrooks, C. J., C. Eldridge, J. Sanes, D. Gottlieb, and R. Bunge. Manuscript in preparation.

We subjected the GuHCl-extracted molecules obtained from N2 and N2FA cultures to gel filtration chromatography on Sepharose CL-4B in 4 M GuHCl buffer. Typical results are shown in Fig. 1. The extracts obtained from N2FA cultures contained one major broad $^{35}SO_4$-containing peak eluting with a K_{av} of 0.21, and a second smaller peak eluting with a K_{av} of 0.44. The N2 material contained $^{35}SO_4$-labeled peaks eluting at the same positions; the relative amounts of the two peaks, however, were different. When calculated per culture, there was ~8 times more radioactivity in the peak of K_{av} = 0.21 in N2FA cultures than in N2 cultures, but only 1.3 times more radioactivity in the peak of K_{av} = 0.44. These results indicate that the increased accumulation of radioactivity in N2FA cultures was due to the selective accumulation of the material of K_{av} = 0.21. Furthermore, significant accumulation of this molecule occurred only when the basal lamina was being formed, whereas accumulation of the material eluted later (K_{av} = 0.44) was independent of basal lamina formation.

Biochemical Characterization

To further analyze these sulfated macromolecules, column fractions were pooled and aliquots of each material were rechromatographed on Sepharose CL-4B in 4 M GuHCl. Additional aliquots were subjected to mild alkaline hydrolysis as described in Materials and Methods and then chromatographed on the same column. Results for the material obtained from the major peak that eluted early from N2FA cultures are shown in Fig. 2. The rechromatographed material eluted at the same position (K_{av} = 0.21) as in the original chromatogram (see Fig. 1), and the alkaline-hydrolyzed material, as a single peak that eluted later (K_{av} = 0.64). Identical results were obtained for the material that eluted early from N2 cultures (not shown). This behavior is what would be expected for a proteoglycan, with the alkaline-hydrolyzed material being glycosaminoglycan chains liberated from the proteoglycan core protein. When this Sepharose CL-4B column was calibrated with chondroitin sulfate glycosaminoglycan chains, a K_{av} of 0.64 was found to correspond to an M_r of 21,000. Similar experiments were done for the material in the second peak (K_{av} = 0.44) obtained from both N2 and N2FA cultures. The rechromatographed material eluted as a single peak with a K_{av} of 0.44, whereas the alkaline-hydrolyzed material eluted as a single peak with a K_{av} of 0.68, corresponding to a glycosaminoglycan of M_r 18,000 (data not shown).

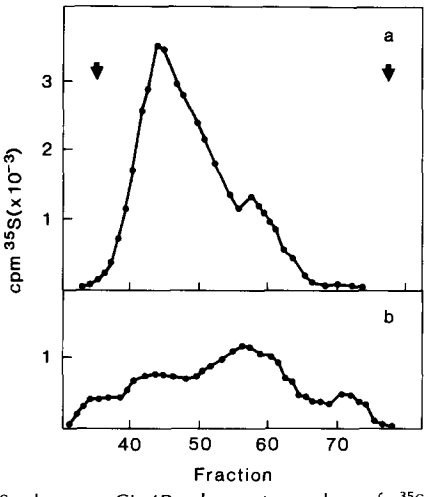

FIGURE 1 Sepharose CL-4B chromatography of $^{35}SO_4$-labeled macromolecules extracted by 4 M GuHCl. 4-wk-old Schwann cell–nerve cell cultures grown in N2 medium were incubated in N2FA (*a*) or N2 medium (*b*) for 2 d and then labeled with $^{35}SO_4$ in Ham's F12 medium with 10 ng/ml nerve growth factor with (*a*) or without (*b*) 50 μg/ml ascorbate and 1 mg/ml fetuin for 48 h. The cells were extracted with 4 M GuHCl as described in Materials and Methods. The graphs represent the radioactivity present in equal numbers of cells. Fraction sizes were 1 ml. Arrows indicate excluded and included volumes.

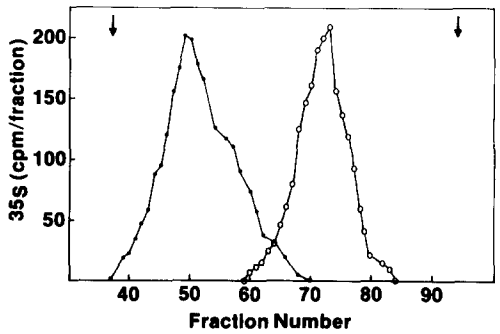

FIGURE 2 Sepharose CL-4B chromatography of sulfated proteoglycan before and after alkaline hydrolysis. The peak of $^{35}SO_4$-labeled material eluting at K_{av} = 0.21 obtained from 4 M GuHCl extracts of N2FA cultures was pooled and rechromatographed on Sepharose CL-4B before (●) and after (○) alkaline/borohydride hydrolysis as described in Materials and Methods. Elution was with 4 M GuHCl buffer; fraction sizes were 0.7 ml. Arrows indicate excluded and included volumes.

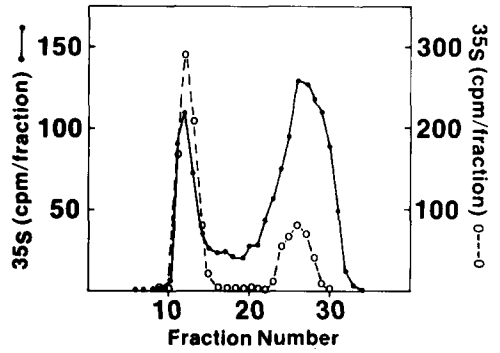

FIGURE 3 Nitrous acid and chondroitinase ABC digestion of alkaline-hydrolyzed sulfated proteoglycan. The peak of $^{35}SO_4$-labeled material eluting at K_{av} = 0.21 obtained from N2FA cultures was subjected to alkaline/borohydride hydrolysis. Aliquots were treated separately with nitrous acid (●) or chondroitinase ABC (○) and then applied to a Sephadex G-50 column. Fraction sizes were 1 ml.

To further support the conclusion that these two molecules are sulfated proteoglycans, aliquots of alkaline-hydrolyzed samples were digested separately with nitrous acid or chondroitinase ABC. In the experiments shown in Fig. 3, ~70% of the $^{35}SO_4$ radioactivity in alkaline-hydrolyzed peak 1 (K_{av} = 0.21) from N2FA cultures was degraded by nitrous acid, and ~30% was degraded by chondroitinase ABC. Identical results were obtained for the material obtained from N2 cultures (not shown). In four such experiments, the range of nitrous acid–degradable $^{35}SO_4$-labeled material was 70–85% (mean 75%). Together with the data presented above, these results indicate that the molecule of K_{av} = 0.21 is a sulfated proteoglycan with glycosaminoglycans of M_r 21,000, approximately three-fourths of which are heparan sulfate (or heparin) and one-fourth of which are chondroitin sulfate (or dermatan

sulfate). Alternatively, this fraction could contain a major heparan sulfate proteoglycan plus contaminating chondroitin sulfate proteoglycans (see below). When similar analyses were carried out for peak 2 (K_{av} = 0.44) alkaline-hydrolyzed material, the results indicated the presence of both heparan sulfate and chondroitin sulfate in a ratio of ~4:1 (data not shown). For convenience, these proteoglycan fractions have been designated PG1 and PG2 for the species eluting early and late, respectively.

To determine whether the heparan sulfate and chondroitin sulfate were attached to the same core protein, $^{35}SO_4$-labeled GuHCl-extracted proteoglycans were digested with chondroitinase ABC and then analyzed by Sepharose CL-4B chromatography. As expected, the enzyme treatment caused a reduction in the amount of radioactivity eluting in the proteoglycan region but did not cause a detectable shift in elution volumes of the proteoglycans (data not shown). This result is consistent with the presence of separate heparan sulfate and chondroitin sulfate proteoglycans.

When 4 M GuHCl extracts of cells were subjected to CsCl equilibrium density gradient centrifugation in the presence of 4 M GuHCl, ~70% of the total macromolecular $^{35}SO_4$, including essentially all of PG1 and PG2, banded at densities <1.4 g/ml (data not shown).

PG1 and PG2 Are Synthesized by Schwann Cells

The collagens and laminin present in the Schwann cell basal lamina are produced by Schwann cells and not by nerve cells (15–17), yet nerve cells are required for basal lamina formation (14). For this reason, we wanted to determine whether Schwann cells, nerve cells, or both, synthesize the two proteoglycans described above. Dorsal root ganglion explant cultures containing Schwann cells and nerve cells were grown in N2FA medium. Just before the cultures were labeled with $^{35}SO_4$, the explant regions were excised, leaving the outgrowth of nerve fibers and associated Schwann cells. Because the excised explants contained all the neuronal cell bodies (the site of nerve cell protein synthesis), any synthesis of labeled proteoglycans detected after removal of the explant must have been due to Schwann cells. Removal of the neuronal cell bodies caused no significant decrease in the amounts of sulfated proteoglycan produced. Furthermore, Sepharose CL-4B profiles of 4 M GuHCl-extracted material obtained from cultures from which the explants had been removed were indistinguishable from those of control cultures labeled with the explant intact (data not shown). From these results, we conclude that PG1 and PG2 are synthesized by Schwann cells and are synthesized by nerve cells either in very small amounts or not at all.

A Mouse Monoclonal Antibody to Basal Lamina Recognizes PG1

Recently, a mouse monoclonal antibody that recognizes a component of the Schwann cell basal lamina has been isolated.[2] This antibody recognizes a heparan sulfate proteoglycan present in the culture medium of Schwann cell–nerve cell cultures; the same proteoglycan is also recognized by antibodies to a tumor-derived basement membrane proteoglycan, BM-1 (see reference 5; and Eldridge, C., personal communication). We tested this monoclonal antibody for its ability to bind in an immunoprecipitation reaction to the cell-associated sulfated proteoglycans produced by Schwann cells. Proteoglycans were extracted with 4 M GuHCl from $^{35}SO_4$-labeled Schwann cell–neuron cultures, dialyzed against 0.05 M Na-phosphate, 0.15 M NaCl, pH 7.4, and then mixed with antibody. After precipitation of the immune complexes, the pellets and supernatants were diluted into 4 M GuHCl buffer and subjected to Sepharose CL-4B chromatography. As shown in Fig. 4, the immunoprecipitate contained only material that eluted as PG1, whereas the supernatant solution contained only material that eluted as PG2. A control immunoprecipitate from a tube incubated without antibody contained no $^{35}SO_4$-containing peaks (Fig. 4). Biochemical analysis of these materials as described above confirmed they were PG1 and PG2 (not shown). From these results, we conclude that the mouse antibody recognizes PG1 but not PG2.

We used this monoclonal antibody to stain by indirect immunofluorescence living, unfixed Schwann cell–nerve cell cultures grown in either N2 or N2FA medium. As shown in Fig. 5, and in agreement with previous results,[2] N2 cultures stain very lightly, if at all, with this antibody. In contrast, cultures induced to form basal lamina by incubation in N2FA medium exhibited bright and specific staining. The pattern of extracellular staining of N2FA cultures resembled qualitatively that of other basal lamina components such as laminin (not shown, but see reference 17) or collagen type IV (Fig. 5, inset; see also reference 16). These results support the conclusion derived from the biochemical studies that PG1 accumulates only during active basal lamina formation.

Effect of Neuronal Deprivation on Schwann Cell Proteoglycan Accumulation

To provide further evidence that the increased accumulation of PG1 in N2FA cultures was due to basal lamina formation and not to other effects of altering the culture medium, we compared the sulfated proteoglycans present in cultures containing both Schwann cells and nerve cells to those in cultures containing only Schwann cells. Schwann cells cultured in the absence of nerve cells do not synthesize new basal lamina (14). For these experiments, Schwann cell–

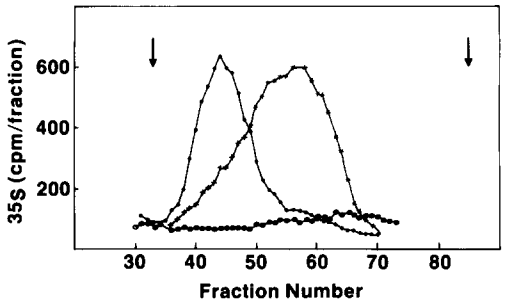

FIGURE 4 Immunoprecipitation of PG1 with a monoclonal antibody. Schwann cell–nerve cell N2FA cultures were labeled with $^{35}SO_4$, and the cells were extracted with 4 M GuHCl. The 4 M GuHCl-extracted macromolecules were dialyzed vs 0.05 M Na-phosphate, 0.15 M NaCl, pH 7.5 and incubated with the mouse monoclonal antibody B3 as described in Materials and Methods. The immunoprecipitated material (●) and supernatant (×) were diluted with 4 M GuHCl buffer and aliquots were applied to a Sepharose CL-4B column and eluted with 4 M GuHCl buffer. A control immunoprecipitate, from an incubation with nonimmune IgG, was analyzed in the same way (○). Fraction sizes were 0.8 ml. Arrows indicate excluded and included volumes.

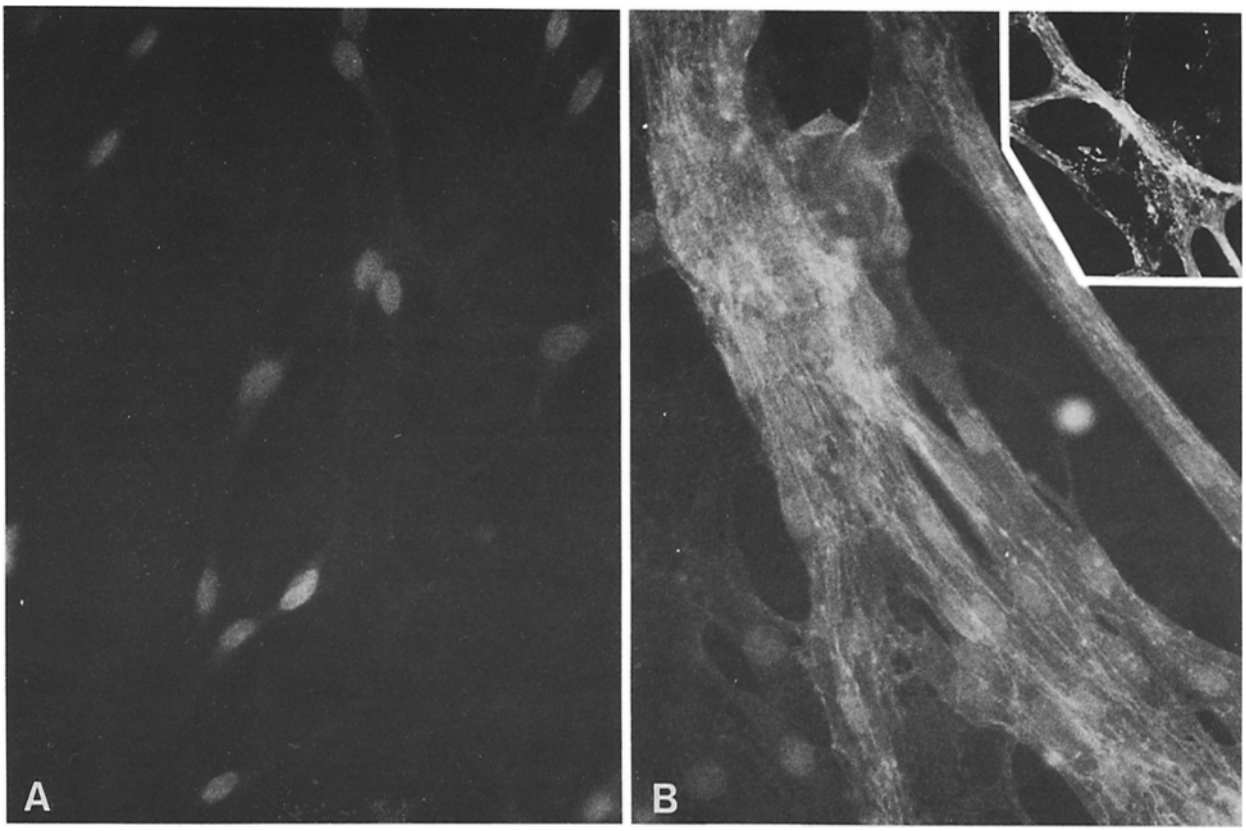

FIGURE 5 Indirect immunofluorescence of Schwann cell–nerve cell cultures with anti-PG1 monoclonal antibody. Cultures grown in N2 for 4 wk were keep in N2 medium (*A*) or switched to N2FA medium (*B*). After 5 d, the cultures were processed for indirect immunofluorescence using the mouse monoclonal antibody B3 which was shown to recognize PG1 (see Fig. 4). The nuclear fluorescence in both micrographs is not due to the antibody (not shown). Note in the N2FA culture (*B*) the linear extracellular staining pattern characteristic of Schwann cell basal lamina staining. The inset in *B* shows an N2FA culture stained with anti-collagen IV antiserum. This antiserum does not stain N2 cultures (not shown). Both specimens were prepared for immunofluorescence, photographed, and printed under identical conditions. × 490.

nerve cell explant cultures were grown for 4 wk in N2FA medium, during which time active basal lamina synthesis occurs. At this time, the ganglia were excised from half the cultures. After this treatment, both groups of cultures were incubated for an additional 1 wk to allow the Schwann cells to phagocytose the axotomized neuritic processes in the outgrowth of ganglion-excised cultures. It has been shown (14) that after neuronal excision, the existing basal lamina persists but no new basal lamina is formed by Schwann cells. 1 wk after excision of the ganglia, the cultures were labeled with $^{35}SO_4$, and the 4 M GuHCl-extracted proteoglycans were analyzed by Sepharose CL-4B chromatography. As shown in Fig. 6, the proteoglycan profile obtained from the nerve cell–containing cultures was typical of N2FA cultures. In contrast, the proteoglycan profile of the Schwann cell cultures more closely resembled that of N2 cultures, with a significantly reduced amount of PG1. This result supports the conclusion that increased accumulation of PG2 occurs only during basal lamina formation.

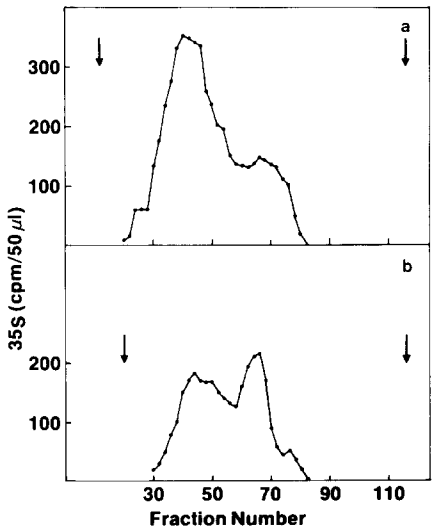

FIGURE 6 Effect of neuronal deprivation on accumulation of Schwann cell proteoglycans. Schwann cell–neuron explant cultures were grown in N2FA medium for 5 wk. The nerve cells were removed from half the cultures by excising the ganglionic explants, and all the cultures were incubated for an additional 1 wk in N2FA medium. At that time, the cultures were labeled with $^{35}SO_4$, and the 4 M GuHCl-extracted proteolglycans were analyzed on Sepharose CL-4B columns. (*a*) Schwann cells with nerve cells; (*b*) Schwann cells only. Fraction sizes were 0.5 ml. Arrows indicate excluded and included volumes.

PG2 but Not PG1 Is Solubilized by Detergent

Some cell-surface heparan sulfate proteoglycans are thought to reside in membranes as integral membrane proteins (10, 12). To determine whether either of the Schwann cell proteoglycans described above might be associated with membranes, we measured the ability of 1% Triton X-100 to extract these proteoglycans from $^{35}SO_4$-labeled cultures. Extraction with

1% Triton X-100 solubilizes most membrane components and extracts soluble proteins but not cytoskeletal or extracellular matrix proteins from these cells (reference 16; and unpublished observations). We observed that the detergent extracted 14–20% (range of three experiments) of the total cell-associated $^{35}SO_4$-labeled proteoglycans in N2FA cultures. Results of gel filtration chromatography on Sepharose CL-4B of the Triton-extracted material is shown in Fig. 7. The major ^{35}S-containing peak eluted at a K_{av} of 0.44. This material was indistinguishable from PG2 based on alkaline hydrolysis, and nitrous acid and chondroitinase ABC digestion (data not shown). In addition, the detergent extracts contained variable amounts of $^{35}SO_4$-labeled material that eluted at a K_{av} of 0.7 (Fig. 7), and that was predominantly free heparan sulfate (data not shown). Only small amounts of material that eluted as PG1 were detected in the detergent extracts. Nearly identical results were obtained for N2 cultures, except that a larger percentage of the total $^{35}SO_4$-labeled macromolecules were extracted by Triton X-100 (28 and 34% in two experiments). This difference reflects the larger proportion of total proteoglycan that is PG2 in these cultures. When Triton-extracted cultures were then extracted with 4 M GuHCl, an additional and approximately equal amount of material similar to PG2 was extracted. Thus, either two proteoglycans with similar properties, one membrane-associated and one not, are made by Schwann cells, or PG2 is membrane-associated but not quantitatively extracted by the conditions used.

DISCUSSION

The results presented demonstrate that Schwann cells synthesize two major heparan sulfate–containing proteoglycans that differ in size and apparent distribution. The larger proteoglycan, which we termed PG1, accumulates only during basal lamina formation and is not extractable by detergent. A monoclonal antibody that recognizes this proteoglycan in an immunoprecipitation reaction stains the outer surface of Schwann cells in a pattern similar to that of other basal lamina components. This antibody does not stain Schwann cells that lack a basal lamina coverage. These observations suggest that PG1 is a component of the Schwann cell basal lamina. Proof of this localization must await immunoelectron microscopic analysis.

The major Schwann cell heparan sulfate proteoglycans as isolated by gel filtration chromatography contain significant amounts of chondroitin sulfate. Digestion of GuHCl-extracted proteoglycans with chondroitinase ABC did not cause a detectable shift in the elution volumes on Sepharose CL-4B of the major Schwann cell proteoglycans, a result consistent with the presence of separate heparan sulfate and chondroitin sulfate proteoglycans. In general, heparan sulfate–containing proteoglycans do not contain other glycosaminoglycans (e.g., 5, 7, 10, 12). Chondroitin sulfate proteoglycans have been shown to be present in peripheral nerves in the Schwann cell basal lamina and surrounding extracellular matrix (28). Interestingly, the glycosaminoglycan composition of $^{35}SO_4$-labeled macromolecules did not differ significantly between N2 and N2FA cultures (not shown). Thus, increased accumulation of one or more chondroitin sulfate proteoglycans may occur along with increased accumulation of PG1. Not surprisingly, fibroblasts obtained from dorsal root ganglia produce large amounts of chondroitin sulfate proteoglycans (unpublished observations). Further experiments will be required to determine the properties of these chondroitin sulfate proteoglycans.

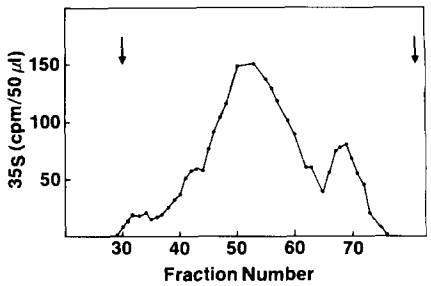

FIGURE 7 Sepharose CL-4B chromatography of Triton X-100–extracted Schwann cell proteoglycans. Schwann cell–nerve cell explant cultures were grown in N2 for 4 wk followed by 3 d in N2FA. The cultures were labeled with $^{35}SO_4$ and extracted with 1% Triton X-100 as described in Materials and Methods. The Triton X-100–extracted macromolecules were diluted with 4 M GuHCl buffer, applied to a Sepharose CL-4B column, and eluted with 4 M GuHCl buffer. Fraction sizes were 0.8 ml. Arrows indicate excluded and included volumes.

Our results indicate the synthesis by Schwann cells of two distinct heparan sulfate proteoglycans. A possibility we cannot entirely rule out, however, is that the smaller proteoglycan, PG2, is a catabolic product of the larger, and that the conversion to PG2 is slowed or prevented in N2FA medium. Several lines of evidence, however, lead us to favor the idea that they are distinct. First, the rate of accumulation of PG2 appears to be independent of culture conditions, and only PG1 accumulation increases when the basal lamina is being formed. While a scheme in which PG2 is formed from PG1 is not in principle inconsistent with this data, we believe the fortuitous result that the amount of PG2 present is unaltered, whereas the amount of PG1 increases dramatically under certain conditions, is unlikely. Second, in preliminary pulse-chase radiolabeling experiments measuring the rates of synthesis and turnover of the Schwann cell proteoglycans, we have found no kinetic evidence for a conversion of PG1 to PG2 (unpublished observations). Finally, the observation that Triton X-100 extracts PG2, but not PG1, while not conclusive, is consistent with these being distinct proteoglycans. A definitive answer to this question will require a detailed analysis of the rates of synthesis and turnover of the proteoglycans under various culture conditions.

Heparan sulfate proteoglycans have been shown to be present in many basal lamina (5–9). Unfortunately, however, detailed information on individual basal lamina proteoglycans is rather scarce, due largely to the difficulties of isolating basal lamina components in pure form with nondegradative techniques. For this reason, basement membrane–producing tumors or cell lines derived from tumors have been widely used. The EHS murine sarcoma and the teratocarcinoma-derived PYS-2 cell line produce structurally distinct but immunologically related heparan sulfate proteoglycans (5, 7). These proteoglycans differ from the Schwann cell basal lamina proteoglycan in apparent overall size and buoyant density. Antibodies to the mouse tumor proteoglycan, however, stain Schwann cells in an indirect immunofluorescence assay (Eldridge, C., personal communication). Thus, although these proteoglycans may differ in their final structure, their core proteins may be similar or identical. Similarly, the isolated perfused rat kidney has been shown to produce two basal lamina–associated proteoglycans, a high buoyant density heparan sulfate proteoglycan, and a low-density chondroitin sulfate proteoglycan (6). Neither of these proteoglycans appear

to be identical to Schwann cell PG1 or the other basal lamina proteoglycans, although antibodies to the mouse tumor proteoglycan also stain the glomerular basement membrane (5). Obviously, more work will be required to clarify the relationship among these basal lamina-associated proteoglycans. The availability of a monoclonal antibody that recognizes the Schwann cell basal lamina proteoglycan should make it possible to isolate and characterize the polypeptide portion of this proteoglycan.

The precise arrangement of PG1 in the basal lamina remains to be determined. The molecular interactions responsible for binding together the components of basal lamina are not completely understood, although several types of interactions have been identified. These include the self-association of collagen type IV molecules (29, 30) and the binding of laminin to other molecules, including collagen type IV, heparan sulfate proteoglycan (4), and a cell surface receptor (31). We do not know whether the Schwann cell basal lamina proteoglycan reported in this study binds to collagen type IV. Experiments to examine the interactions among the Schwann cell basal lamina components are in progress.

In contrast to PG1, the smaller Schwann cell-produced proteoglycan, PG2, is present in significant amounts in cultures lacking basal lamina. A significant fraction of this proteoglycan (up to one-half) is extracted by Triton X-100, which solubilizes cell membranes but not the Schwann cell basal lamina. These results suggest that PG2 is associated with cell membranes, either directly as an integral membrane protein, or by its binding to an integral membrane component. Examples of both types of membrane interaction exist for proteoglycans in other tissues (10, 12). The inability to extract all of the PG2 with Triton X-100 indicates either that there are two similar proteoglycans with different localizations within the cell or there is a single proteoglycan, a portion of which is bound to detergent-insoluble cellular structures. The observation that the detergent extractability of PG2 was not altered by the presence or absence of basal lamina suggests it is not bound to extracellular matrix molecules. A possible explanation is that PG2 binds to intracellular cytoskeletal structures. Preliminary evidence for an interaction between the cytoskeleton and a membrane proteoglycan in mammary epithelial cells has been published (32). Experiments to further characterize this proteoglycan and the nature of its association with the Schwann cell membrane are in progress.

The functions of these proteoglycans are not known. Proteoglycans and glycosaminoglycans in other tissues have been implicated in such processes as tissue morphogenesis (2, 21, 23), cell migration (2), and maintenance of a permeability barrier (20). In the context of nervous system development, there is evidence that a heparan sulfate proteoglycan produced by bovine corneal endothelial cells promotes neurite growth from cultured neurons (22). The availability of the Schwann cell–nerve cell culture system should allow us to begin to probe the functions of these molecules in such cellular processes as matrix assembly, neurite growth, Schwann cell proliferation and migration, and ensheathment and myelination of nerve fibers.

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