

# GAP-43 Is Expressed by Nonmyelin-forming Schwann Cells of the Peripheral Nervous System

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**Abstract.** Recently it has been demonstrated that the growth-associated protein GAP-43 is not confined to neurons but is also expressed by certain central nervous system glial cells in tissue culture and *in vivo*. This study has extended these observations to the major class of glial cells in the peripheral nervous system, Schwann cells. Using immunohistochemical techniques, we show that GAP-43 immunoreactivity is present in Schwann cell precursors and in mature nonmyelin-forming Schwann cells both *in vitro* and *in vivo*. This immunoreactivity is shown by Western blotting to be a membrane-associated protein that comigrates with purified central nervous system GAP-43. Furthermore, metabolic labeling experiments demonstrate definitively that Schwann cells in culture can synthesize GAP-43. Mature myelin-forming Schwann

cells do not express GAP-43 but when Schwann cells are removed from axonal contact *in vivo* by nerve transection GAP-43 expression is upregulated in nearly all Schwann cells of the distal stump by 4 wk after denervation. In contrast, in cultured Schwann cells GAP-43 is not rapidly upregulated in cells that have been making myelin *in vivo*. Thus the regulation of GAP-43 appears to be complex and different from that of other proteins associated with nonmyelin-forming Schwann cells such as N-CAM, glial fibrillary acidic protein, A5E3, and nerve growth factor receptor, which are rapidly upregulated in myelin-forming cells after loss of axonal contact. These observations suggest that GAP-43 may play a more general role in the nervous system than previously supposed.

**GROWTH-ASSOCIATED** protein-43 (GAP-43,<sup>1</sup> otherwise known as F1, B-50, neuromodulin, and pp46) was first identified as a membrane phosphoprotein in neurons (for review see Skene, 1989). GAP-43 is synthesized at high levels during axonal outgrowth in development or regeneration (Skene and Willard, 1981a,b) and then transported to the growth cone where it associates with both the cytoskeleton and plasma membrane at sites of substrate adhesion (Skene et al., 1986; Meiri et al., 1986; Meiri and Gordon-Weeks, 1990). GAP-43 remains detectable in the adult rat brain in areas of continuing synaptic turnover (Benowitz and Routtenberg, 1987) and in some axon tracts (Gorgels et al., 1989), but synthesis declines an order of magni-

tude after synaptogenesis (Jacobson et al., 1986). GAP-43 is commonly referred to as a "neuron-specific protein," since it has frequently been suggested that its major function is involvement in axon motility or axon elongation especially at the growth cone (e.g., Benowitz and Routtenberg, 1987). Recent evidence has shown, however, that GAP-43 is also expressed by some glial cells in the central nervous system (CNS), raising new questions about the role of this protein. In these studies, GAP-43 was found in bipolar oligodendrocyte/type 2 astrocyte progenitors, type 2 astrocytes, and immature oligodendrocytes (Vitković et al., 1988; da Cunha and Vitković, 1990; Deloulme et al., 1990), although this was downregulated in oligodendrocytes as they matured *in vitro* and *in vivo* (Curtis et al., 1991).

The presence of GAP-43 in the glial cells of the peripheral nerve, the myelin-, and nonmyelin-forming Schwann cells, is at present controversial. Tetzlaff et al. (1989) demonstrated the presence of GAP-43 immunoreactivity in reactive Schwann cells of the regenerating facial and sciatic nerves but it was concluded from these experiments that the GAP-43 had been taken up from the regenerating axons by an unknown mechanism. Likewise, Woolf et al. (1990), using cultures of dorsal root ganglia, showed that some Schwann/satellite cells ex-

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1. Abbreviations used in this paper: CNS, central nervous system; E15, embryonic day 15; GAP-43, growth-associated protein-43; GFAP, glial fibrillary acidic protein; MBP, myelin basic protein; NGF, nerve growth factor.

pressed GAP-43, albeit at low levels. Again, the possibility of GAP-43 uptake from the neurons in the culture could not be eliminated. In contrast, no GAP-43 immunoreactivity was detected in pure Schwann cells or in sympathetic neuron/Schwann cell mixed cultures (Meiri et al., 1988). Furthermore, the studies of Verhaagen et al. (1986) failed to detect any immunoreactive GAP-43 in intact rat sciatic nerve or in the distal stump after nerve transection, and Fitzgerald et al. (1991) detected no neuroglial GAP-43 in sections of developing nerves.

To resolve this issue of Schwann cell GAP-43 expression we have used metabolic labeling, immunohistochemistry, and Western blotting techniques. Using a polyclonal antiserum raised against  $\beta$ -galactosidase/GAP-43 fusion protein (Curtis et al., 1991), we have evaluated GAP-43 staining in conjunction with antigenic markers of both myelin and non-myelin-forming Schwann cells in the normal sciatic nerve and in the cervical sympathetic trunk (in which 99% of the axons are unmyelinated; Aguayo et al., 1976a). GAP-43 expression was also examined in Schwann cell precursors and Schwann cells in vitro and in the sciatic nerve after transection, allowing analysis of Schwann cells in the absence of an axonal source of GAP-43 and determination of the temporal changes in GAP-43 expression after the removal of Schwann cells from axonal contact. Furthermore, using metabolic labeling and immunoprecipitation techniques we definitively demonstrate that Schwann cells in culture can synthesize GAP-43.

On the basis of these studies, we conclude that Schwann cell precursors and nonmyelin-forming Schwann cells in the normal mature peripheral nervous system can express GAP-43 and that Schwann cells in the absence of axonal contact can synthesize GAP-43. Proliferating Schwann cells reacting to loss of axonal contact after surgical denervation express GAP-43, but this is delayed relative to the appearance of other markers of the nonmyelin-forming phenotype. Immunoblotting of proteins from sympathetic trunk and chronically denervated sciatic nerve and membranes prepared from cultured Schwann cells revealed that the immunoreactivity comigrated with GAP-43 purified from rat CNS, showing that Schwann cells synthesize GAP-43, which is the same as the neuronal protein, at least with respect to molecular weight and membrane attachment.

## Materials and Methods

### Animals

Sprague Dawley rats were used throughout. Embryos were removed from females at 15 d of gestation (embryonic day 15, or E15) after killing with carbon dioxide inhalation. Neonates were killed by decapitation and adults of  $\sim 200$  g were killed with either carbon dioxide or pentobarbital sodium and exsanguinated. In some adult animals the sciatic nerve was aseptically exposed at mid-thigh level under halothane anesthetic and cut. After transection, the proximal stump was deflected and embedded in nearby muscle to prevent sprouting axons reaching the distal stump. The absence of axons was confirmed immunohistochemically upon sacrifice after recovery periods of up to 60 d.

### Cell Suspensions

Schwann cells were prepared using a modification of the method of Brockes et al. (1979). Briefly, sciatic nerves were dissected from 1-d-old pups and E15 embryos and the epineurial sheaths were removed wherever possible. The tissue was finely chopped in 0.4% collagenase and 0.25% trypsin in

calcium- and magnesium-free DME and then digested at 37°C in air containing 5% CO<sub>2</sub> for 30 min, followed by trituration through a 200- $\mu$ l pipette tip (Gilson Co., Inc., Worthington, OH). Cells were then centrifuged at 500 g for 10 min at 4°C and resuspended in DME containing 10% calf serum.

Cell suspensions were also prepared from adult cervical sympathetic trunk. To increase the dissociation of adult tissue, enzymatic digestion was extended to 90 min.

### Dried Cell Preparations

Freshly dissociated Schwann cells were resuspended in a minimal volume (25–100  $\mu$ l). 5–20  $\mu$ l was spread on gelatin-coated slides with a plastic pipette tip and allowed to dry for a minimum of 2 h before immunostaining.

### Schwann Cell Cultures

Schwann cells prepared from four 1-d-old rats were maintained in 25-cm<sup>2</sup> tissue culture flasks in water-saturated air containing 5% CO<sub>2</sub> at 37°C. Medium was DME with 10% calf serum, supplemented with 10<sup>-5</sup> M cytosine arabinoside to kill rapidly dividing fibroblasts (Brockes et al., 1979). After 3 d, an enriched population of Schwann cells (approximately one million cells) was recovered by trypsinization and centrifugation at 500 g. These cells were either replated on laminin-coated 13-mm-diam coverslips (20,000 cells per coverslip) for immunostaining or processed for Western blotting (see below). Primary Schwann cell cultures used for immunoprecipitations were maintained in 55-cm<sup>2</sup> laminin-coated tissue culture dishes.

### Embryonic Sensory Neuron Cultures

The dorsal root ganglia from E18 rats were digested as described above for 90 min. Neurons were plated at  $\sim 10^5$  per dish in laminin-coated 35-mm<sup>2</sup> tissue culture dishes in DME containing 7.5% horse serum, 7.5% FCS, and nerve growth factor (NGF). 10<sup>-5</sup> M cytosine arabinoside was added to the cultures for the first 3 d to kill rapidly dividing cells. Subsequently, the medium was changed to fresh medium without cytosine arabinoside for 4 d, and then replaced again with fresh medium containing cytosine arabinoside for an additional 6 d after which time the cultures were used for immunoprecipitation.

### Teased Nerve Preparations

The distal segments of lesioned sciatic nerves and sympathetic trunk and sciatic nerve from normal adult rats were excised and desheathed. The nerves were split into manageable strands and then teased gently onto gelatin-coated microscope slides in a drop of PBS using 23-gauge needles as previously described (Jessen and Mirsky, 1984). The teased nerves were allowed to air dry for at least 2 h before immunofluorescent labeling.

### Cryostat Sections

Normal sciatic nerves and distal portions of lesioned nerves were excised and immediately frozen on cork blocks embedded in Tissue-Tek (Miles Laboratories, Ltd., Slough, UK). 5- $\mu$ m sections were cut at -20°C in a Cryocut E (Reichert Jung S.A., Paris) and thaw mounted onto gelatin-coated slides. After air drying for at least 2 h, the sections were immunostained.

### Immunohistochemistry

Rabbit antiserum to  $\beta$ -galactosidase/GAP-43 fusion protein has been described previously (Curtis et al., 1991).

Cultured Schwann cells were lightly fixed with 2% paraformaldehyde for 5 min before staining with 192-IgG mAb against NGF receptor, diluted 1:5,000 (Taniuchi et al., 1986). Bound antibodies were visualized with TRITC-goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) before further fixation with 4% paraformaldehyde for 15 min and permeabilization in methanol at -20°C for 10 min. Cells were then double-labeled with rabbit anti-GAP-43 diluted 1:1,000 and FITC-goat anti-rabbit IgG (Cappel Laboratories). All antibody incubations were for 30 min.

Teased nerves and dried cells were fixed in 4% paraformaldehyde for 20 min and permeabilized in methanol for 10 min at -20°C. Cryostat sections were fixed with 4% paraformaldehyde for 20 min, and then autofluorescence was quenched with 0.1% sodium borohydride for 5 min. Tissue sections and teased nerves were stained overnight with rabbit anti-GAP-43 diluted 1:5,000 simultaneously with mAbs against vimentin (DAKO, High

Wycombe, UK; 1:10), glial fibrillary acidic protein (GFAP; Boehringer Mannheim, Lewes, UK; 1:10), neurofilament (Anderton et al., 1982; 1:1,000), or myelin basic protein (MBP; Raible and McMorris, 1989; 1:1,000). In some experiments, 91E12 anti-GAP-43 mAb (Goslin et al., 1988) and rabbit polyclonal anti-GFAP (DAKO) were used. Dried cells were stained overnight with rabbit anti-GAP-43 diluted 1:1,000. Controls were performed by omission of the primary antibody or substitution of the primary antibody with preimmune serum. TRITC-goat anti-rabbit IgG (Cappel Laboratories) was applied for 1 h simultaneously with either FITC-goat anti-mouse IgG1 (Nordic Immunology, Tilburg, The Netherlands) or biotin-goat anti-mouse IgG1 (Serotec, Oxford, UK) followed by FITC-streptavidin (Serotec) (for GFAP and 91E12 mAbs).

### Analysis of Proteins by Western Blotting

GAP-43 was purified from CNS by the method of Chan et al. (1986) with the addition of calmodulin affinity chromatography (Andreasen et al., 1983).

Schwann cell membranes were prepared at 4°C in 0.32 M sucrose buffered with 10 mM Hepes (pH 7.5). Protease inhibitors (2 µg/ml leupeptin and 1 mM PMSF) were used throughout. Schwann cells were sheared through a ball-bearing cell disrupter with a 12-µm clearance (Balch et al., 1984). The postnuclear supernatant was centrifuged at 100,000 g to pellet the membranes which were immediately solubilized in SDS solubilization buffer (2% SDS, 2 mM EDTA, 2 mM EGTA, 5 mM Tris, pH 6.8) and protein was estimated (Bio-Rad, Watford, UK). Approximately 10 µg was subjected to 10% SDS-PAGE (Laemmli, 1970).

Denervated sciatic nerve (28 d after transection) and normal cervical sympathetic trunk and ventral root were homogenized directly in SDS solubilization buffer; the protein concentrations were estimated; and 15 µg denervated sciatic nerve, 5 µg sympathetic trunk, and 5 µg ventral root were subjected to 10% SDS-PAGE as above.

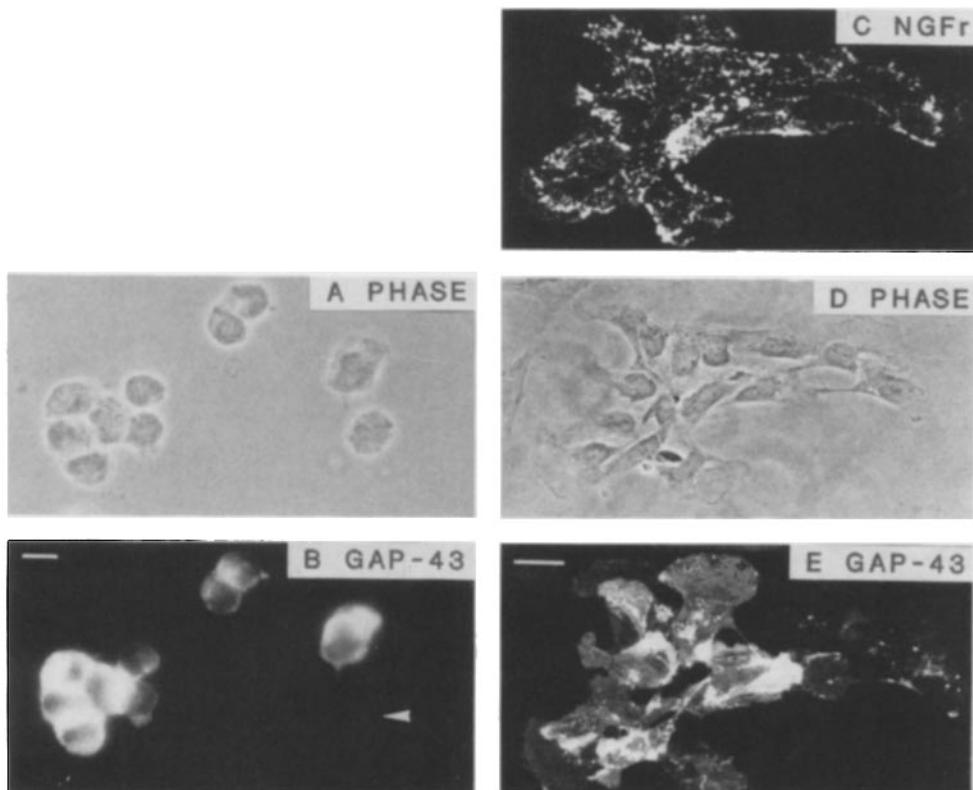
Separated proteins and purified GAP-43 were transferred onto nitrocellulose paper, which was blocked with 10% FCS in 25 mM Tris/saline (pH 7.4) and stained overnight with rabbit anti-GAP-43 diluted 1:1,000 in the same buffer. Immunoreactive bands were visualized with HRP-conjugated swine anti-rabbit IgG (DAKO) using chloronaphthol as chromogen or HRP-conjugated donkey anti-rabbit Ig (Amersham International, Amersham, UK) using DAB as chromogen. Preimmune serum at the same concentration served as a negative control.

### Immunoprecipitation

Immunoprecipitation of GAP-43 was carried out on primary Schwann cell cultures from neonatal rats and on sensory neurons prepared from embryonic day 18 rat dorsal root ganglia (see above). Cultures were washed three times in minimum essential medium containing 0.2 M Hepes (MEMH), and then incubated for 30 min in MEM containing Earle's salts without cysteine and methionine (Gibco, Paisley, UK). Approximately  $1.25 \times 10^6$  Schwann cells were incubated for 4 h at 37°C in 2 ml of methionine- and cysteine-free medium containing 3.5 Mbq ml<sup>-1</sup> of Trans <sup>35</sup>S-label L-methionine (0.1 mCi ml<sup>-1</sup>) (Flow Laboratories, Ayrshire, UK). Neuronal cultures containing  $\sim 10^5$  neurons were incubated in 0.5 ml of the same medium. Cells were then washed rapidly three times in ice cold MEMH and extracted for 30 min on ice with 1 ml of lysis buffer (10 mM Tris-Cl, 0.15 M NaCl, 1 mM EDTA, pH 7.4, containing 1% NP-40 and 1 mM PMSF). After spinning the extract for 30 min at 14,000 g the supernatant was removed and incubated first with 100 µl of protein A linked to agarose beads (Sigma Chemical Co., Poole, UK) to remove nonspecific precipitates, and then with 2 µl of rabbit polyclonal anti-GAP-43 or with normal rabbit serum followed by protein A beads to specifically immunoprecipitate. Pellets were washed thoroughly, boiled in SDS solubilization buffer, and subjected to 10% SDS-PAGE as described above. After running, gels were impregnated with Amplify (Amersham International), dried, and placed in contact with pre-flashed X-ray film for 5 d at -70°C in a cassette equipped with an intensifying screen.

### Results

Previous studies have suggested that some Schwann cells contain GAP-43 in the presence of axons and it has been proposed that Schwann cells might take up the protein from a neuronal source (Tetzlaff et al., 1989; Woolf et al., 1990). The results presented here show that GAP-43 is synthesized by Schwann cells in the absence of axons, either in tissue culture or in the chronically denervated distal stump of transected sciatic nerve. Furthermore, we show that Schwann



**Figure 1.** E15 Schwann cell precursors express GAP-43. (A and B) Dried dissociated sciatic nerve preparations show that most Schwann cell precursors express GAP-43. A single GAP-43<sup>-</sup> precursor (arrowhead) is shown in B. (C-E) Precursor Schwann cells cultured for 24 h, identified by the presence of NGF receptor (C), continue to express GAP-43 (E). Bars, 10 µm.

cell precursors express GAP-43 and that during development GAP-43 becomes restricted to the nonmyelin-forming cells. After denervation, most Schwann cells begin to synthesize GAP-43 with a delay of 2–4 wk.

#### **Schwann Cell Precursors and Nonmyelin-forming Adult Schwann Cells Are Immunoreactive for GAP-43**

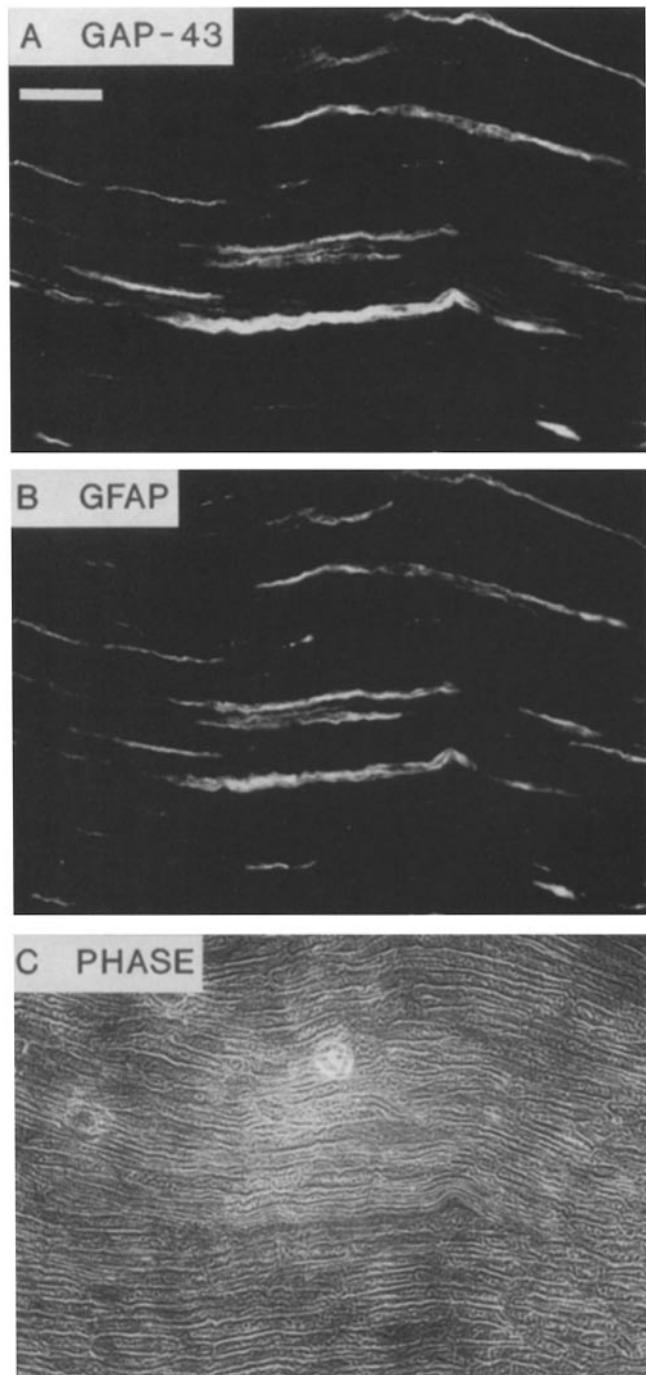
To study GAP-43 expression in Schwann cell precursors, we examined freshly dissociated cell preparations from E15 sciatic nerves. Essentially all the cells (96%) were strongly immunolabeled by antibodies against GAP-43 (Fig. 1, *a* and *b*). Since the large majority of cells in these preparations are precursor cells (Jessen and Mirsky, 1991) this indicates that Schwann cell precursors (NGF receptor<sup>+</sup>, S100<sup>-</sup>) express GAP-43 in vivo. Embryonic Schwann cells continue to express GAP-43 after 24 h in culture (Fig. 1, *c–e*).

GAP-43 expression in mature Schwann cells was initially examined in frozen sections of normal adult sciatic nerve which were double labeled with mAbs against MBP or GFAP to identify myelin-forming and nonmyelin-forming Schwann cells, respectively. Fig. 2 shows that GAP-43 codistributed with GFAP immunoreactivity suggesting that GAP-43 was present in nonmyelin-forming Schwann cells. GAP-43 was not detected in those cells containing MBP (not shown). To examine this further we used teased preparations of the sciatic nerve (Fig. 3) and the cervical sympathetic trunk (Fig. 4). GAP-43 was present in the GFAP<sup>+</sup> nonmyelin-forming Schwann cells, but absent from those cells expressing MBP, confirming that in adult nerves in vivo GAP-43 is restricted to nonmyelin-forming Schwann cells. Similar results were obtained from the vagus nerve (data not shown). This immunostaining was not due to a cross-reacting protein as Western blotting of whole sympathetic trunk revealed a single immunoreactive band which comigrated with GAP-43 purified from CNS (Fig. 5). Western blotting of the largely myelinated ventral root failed to reveal any GAP-43 immunoreactivity (Fig. 5), further demonstrating that GAP-43 is not expressed by myelin-forming Schwann cells.

Despite the distribution of immunostaining apparently outlining the Schwann cells in teased nerves and frozen sections, we wished to exclude the possibility that axons ensheathed by the Schwann cells were responsible for the staining. For this purpose we enzymatically dissociated the adult sympathetic trunk and dried the cell suspension on gelatin-coated microscope slides before double immunolabeling with GAP-43 antibodies and GFAP mAb, to identify nonmyelin-forming Schwann cells. In these preparations, GAP-43 immunoreactivity could be clearly detected in 33% ± 4.7% ( $n = 3$ ) of the GFAP<sup>+</sup> Schwann cells. Possible reasons for the disparity between the teased nerve and dried cell preparations are discussed below.

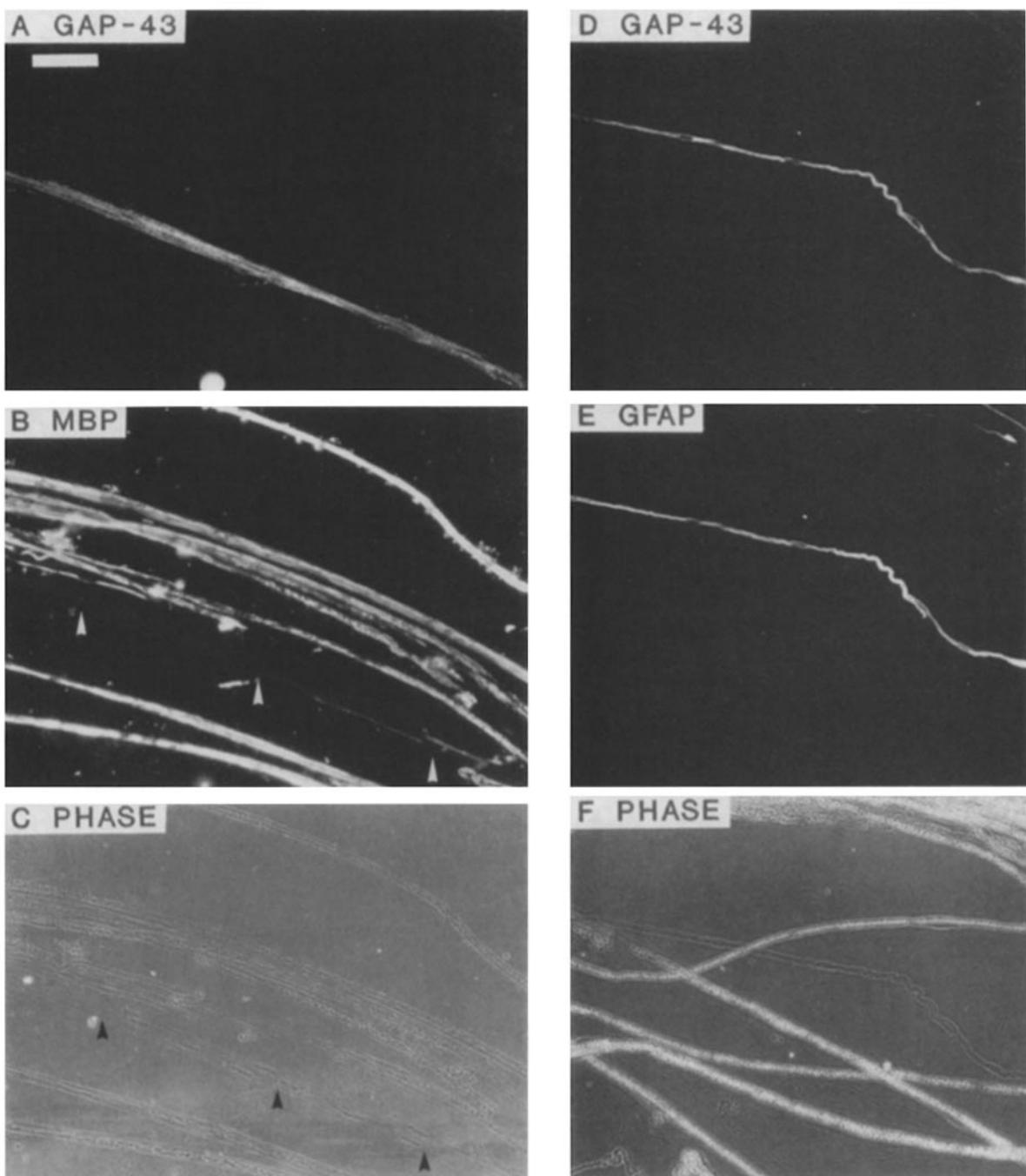
#### **GAP-43 Is Expressed and Synthesized by Schwann Cells In Vitro**

Cultures were prepared from neonatal sciatic nerves and used for immunocytochemistry and as a source of Schwann cell membranes for Western blotting. In tissue culture, at all time points tested between 3 h and 3 wk, 25–40% of Schwann cells from postnatal day 1 rats were GAP-43 immunoreactive (Fig. 6). The persistence of GAP-43 immunoreactivity in these cells shows that neither uptake from axons nor residual axonal segments could be responsible for



**Figure 2.** Nonmyelin-forming Schwann cells express GAP-43 in frozen sections of normal adult sciatic nerve. GAP-43 (*A*) colocalizes with GFAP (*B*), a marker for nonmyelin-forming Schwann cells, in these sections. Bar, 50  $\mu$ m.

the GAP-43 staining. The observation that GAP-43 was not detectable in more than half of the cultured Schwann cells, even after several days in vitro, indicates that the regulation of GAP-43 expression differs from that of other markers of nonmyelin-forming Schwann cells such as GFAP and NGF receptor. These molecules are rapidly upregulated in the great majority of previously myelin-forming Schwann cells after the loss of axonal contact in tissue culture (Mirsky and Jessen, 1991). GAP-43 immunoreactivity was also occasion-



**Figure 3.** GAP-43 is not present in myelinating Schwann cells of normal adult sciatic nerve. Teased sciatic nerve preparations were double-labeled with anti-GAP-43 (*A* and *D*) and mAbs against MBP (*B*) or GFAP (*E*). (*A–C*) Myelin-forming Schwann cells, identified by the presence of MBP, do not contain GAP-43. Only a cable of nonmyelin-forming cells (arrowheads) express GAP-43. (*D–F*) Phase-contrast image (*F*) shows the presence of myelinating Schwann cells which do not express GAP-43, while a cable of GFAP<sup>+</sup> nonmyelin-forming cells are GAP-43<sup>+</sup>. Bar, 50  $\mu$ m.

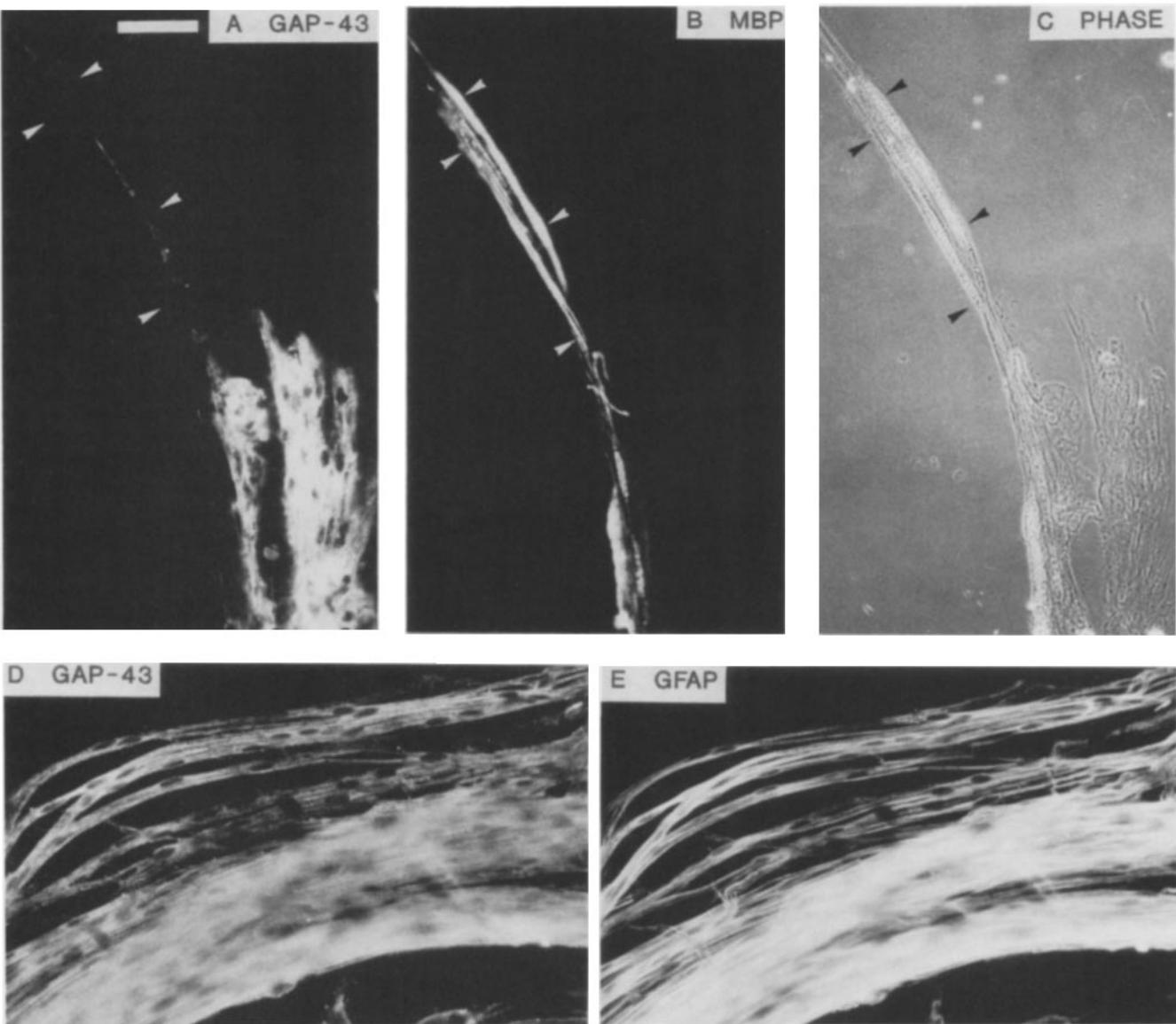
ally detected in fibroblasts contaminating these Schwann cell cultures (data not shown).

Western blots of Schwann cell membrane preparations revealed a single immunoreactive protein band that comigrated with CNS GAP-43 (Fig. 5).

Metabolic labeling of purified Schwann cell cultures with [<sup>35</sup>S]methionine confirmed that Schwann cells can synthesize GAP-43 in vitro (Fig. 5).

#### GAP-43 Is Upregulated during Long-Term Denervation

To investigate GAP-43 expression in Schwann cells deprived of axons *in vivo*, the sciatic nerve was transected and reinnervation was prevented. The absence of regenerating axons in the distal stump was determined by staining teased preparations and cryostat sections with the RT97 mAb which recognizes the phosphorylated form of the 210-kD neurofila-



**Figure 4.** All nonmyelin-forming Schwann cells in the essentially unmyelinated cervical sympathetic trunk express GAP-43. Teased sympathetic trunk preparations were double-labeled with anti-GAP-43 (*A* and *D*) and mAbs against MBP (*B*) or GFAP (*E*). GAP-43 is expressed by all GFAP<sup>+</sup> nonmyelin-forming Schwann cells (*D* and *E*), while the few MBP<sup>+</sup> myelin-forming cells present (arrowheads in *A-C*) do not contain GAP-43. Bar, 50  $\mu$ m.

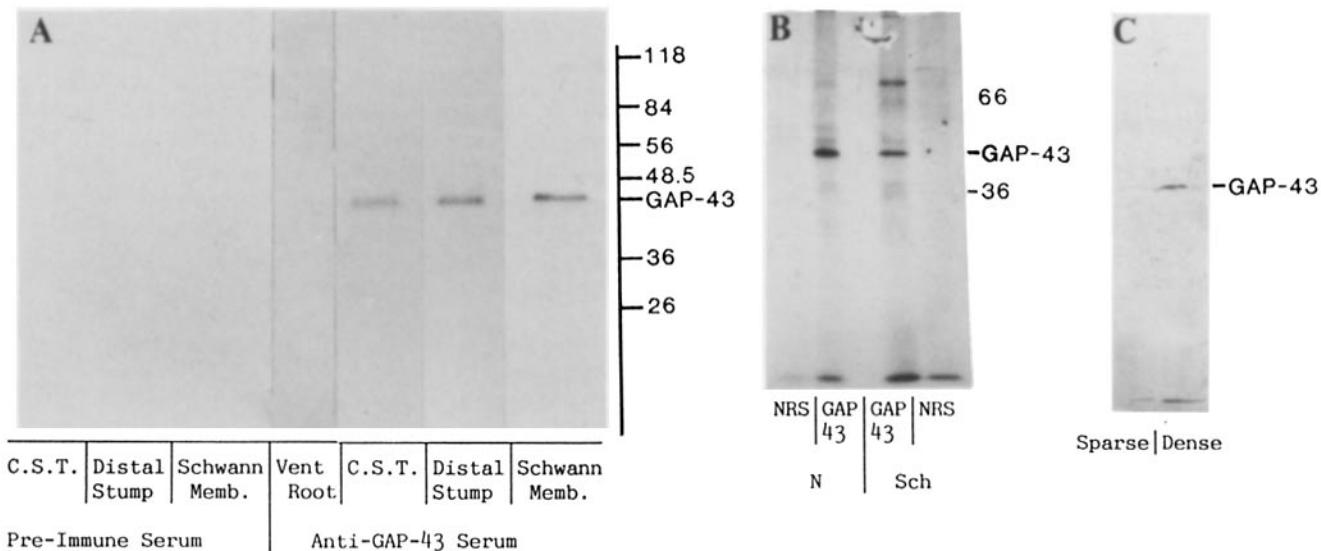
ment protein. In preparations from normal sciatic nerve, RT97 stained both myelinated axons and bundles of unmyelinated fibers (data not shown).

Permanent denervation did not produce an immediate increase in the number of Schwann cells expressing GAP-43; however, almost the entire population appeared immunoreactive by 4 wk in teased distal nerve segments (Fig. 7). A similar pattern of delayed upregulation could also be seen in frozen sections from denervated distal stump (Fig. 7). This was not due to expression by macrophages as GAP-43 did not colocalize with ED1, a marker of this cell type (data not shown). In view of the long-term absence of axons it is highly unlikely that a neuronal source of GAP-43 could be responsible for the GAP-43 immunoreactivity, either directly or by uptake of GAP-43 by Schwann cells from axons.

To confirm that the antiserum was recognizing GAP-43 in

these reactive Schwann cells, distal stumps of sciatic nerve were homogenized 4 wk after transection. Western blotting showed a single band which comigrated with purified GAP-43 from CNS (Fig. 5). The possibility of axonal ingrowth into the tissue used for Western blotting was excluded by RT97 staining of the proximal portion of the distal stump to reveal the presence of regenerating fibers; none were found (data not shown).

Although GAP-43 was expressed by essentially all Schwann cells in the distal stump of cut nerves, only a fraction of Schwann cells cultured in the absence of neurons were GAP-43<sup>+</sup> (previous section). This indicated that factors other than the presence or absence of axons might take part in regulating GAP-43 expression in Schwann cells. Autocrine or paracrine interactions represent a category of potential regulatory mechanisms that are likely to be more effective



**Figure 5.** (A) Anti-GAP-43 antiserum recognizes only GAP-43 in Western blots of purified membranes from cultured Schwann cells (*Schwann Memb.*) and whole tissue homogenates of cervical sympathetic trunk (*C.S.T.*) and denervated distal stump of sciatic nerve 4 wk after transection (*Distal Stump*). No detectable GAP-43 was found in homogenates from ventral root (*Vent Root*). Preimmune serum did not recognize any proteins in these preparations. Relative molecular mass (in kD) of standard proteins and the position of purified CNS GAP-43 are indicated to the right. (B) Immunoprecipitation with anti-GAP-43 antiserum (GAP-43) reveals a band at the GAP-43 position in the [ $^{35}$ S]methionine-labeled lysates from both neuronal (*N*) and Schwann cell (*Sch*) cultures separated by SDS-PAGE and exposed to autoradiographic film. This band is absent in lysates treated with normal rabbit serum (*NRS*), demonstrating that, like neurons, Schwann cells can synthesize GAP-43. (C) Anti-GAP-43 antiserum reveals a clear band of GAP-43 in Western blots of Schwann cells grown at 40,000 cells per  $\text{cm}^2$  (*Dense*), whereas in cells grown at 13,300 cells per  $\text{cm}^2$  (*Sparse*) the band is barely visible. In both cases 10  $\mu\text{g}$  of protein was loaded on to the gel. This suggests that GAP-43 synthesis in Schwann cells depends at least in part on cell density.

in the confined environment of Schwann cells present in the distal stump than in dispersed cell cultures. We therefore tested whether growing Schwann cells at greatly increased cell density would promote GAP-43 expression. When Schwann cell cultures were plated more densely, increased levels of GAP-43 immunoreactivity were seen in cultures examined immunohistochemically. This observation was confirmed by comparing GAP-43 levels in Schwann cells plated at a density of 40,000 cells per  $\text{cm}^2$  and 13,300 cells per  $\text{cm}^2$  (Fig. 5) by Western blotting. Cells plated at the higher density clearly expressed more GAP-43 than those plated at the lower density.

## Discussion

The data presented here demonstrate that GAP-43 is present in essentially all Schwann cell precursors and in mature nonmyelin-forming Schwann cells, but is downregulated in mature myelin-forming Schwann cells. Western blots of cells in culture and denervated distal stumps of transected nerves show that Schwann cells in the absence of axons synthesize GAP-43 of the same molecular weight as protein purified from the CNS.

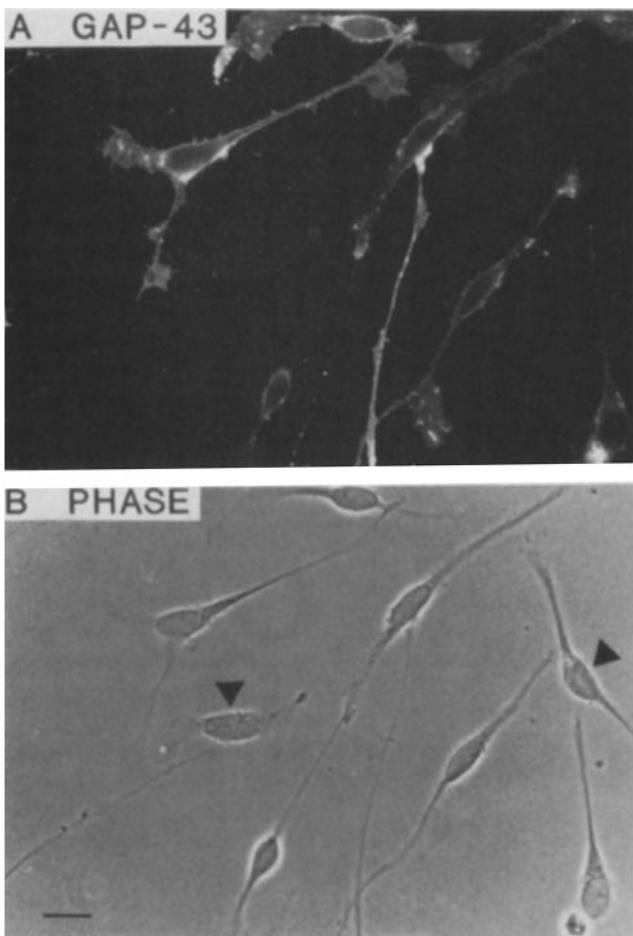
### GAP-43 Is Present in Schwann Cell Precursors Early in Development and in Mature Nonmyelin-forming Schwann Cells

GAP-43 is detectable in 96% of all cells in dried preparations from E15 sciatic nerve. Most cells in the sciatic nerve at this stage are Schwann cell precursors, lacking the S100 protein

which defines Schwann cells (Jessen and Mirsky, 1991). GAP-43 is therefore an early marker of the Schwann cell lineage.

Immature Schwann cells in developing peripheral nerves are directed to become myelin-forming by contact with large-diameter axons, with which they form a one-to-one relationship. Axonal contact specifying the myelinating pathway also causes downregulation of certain proteins present in the immature Schwann cells including GFAP, N-CAM, A5E3, Ran-2, and NGF receptor. However, these proteins persist in the remaining Schwann cells and can be used as markers to define the mature nonmyelin-forming phenotype (Jessen et al., 1990). In teased nerve preparations from mixed nerves (vagus and sciatic) and the essentially unmyelinated cervical sympathetic trunk, GAP-43 colocalized with GFAP<sup>+</sup> nonmyelin-forming Schwann cells and could not be seen in myelinating Schwann cells that were identified by MBP expression. Furthermore the lack of detectable GAP-43 in Western blots of the highly myelinated ventral root confirms these observations. Thus, GAP-43 appears to join the growing list of protein markers for nonmyelin-forming Schwann cells (Jessen et al., 1987; Jessen et al., 1990).

It is not clear whether all nonmyelin-forming Schwann cells express GAP-43. In the sympathetic trunk only 33% of nonmyelin-forming Schwann cells were stained in dissociated cell preparations whereas in teased nerve preparations GAP-43 immunoreactivity appeared associated with nearly all Schwann cells. Likewise, GAP-43 was difficult to detect in dissociated cells from normal and lesioned sciatic nerves, despite the fact that intense immunostaining was seen in Schwann cells in tissue sections and teased prepara-



**Figure 6.** A subpopulation of Schwann cells expresses GAP-43 in tissue culture. A cluster of GAP-43<sup>+</sup> Schwann cells (*A*) are visible in a culture prepared from a 1-d-old rat, maintained in vitro for 24 h. Phase-contrast image (*B*) shows in addition some cells that do not contain GAP-43 (arrowheads). The majority of Schwann cells were GAP-43<sup>-</sup> up to 3 wk in vitro (see text). Bar, 10  $\mu$ m.

tions of chronically denervated distal nerve segments where no axons were present. We do not currently understand why GAP-43, an intracellular protein, should be lost during the preparation of these cells, but it may depend on loss of GAP-43 from the plasma membrane by depalmitoylation (Skene and Virág, 1989), followed by leaching of the soluble protein from the cytoplasm during permeabilization and fixation. These problems clearly do not occur in cells dissociated from embryonic nerves, where nearly all the isolated cells contained GAP-43.

Since peripheral axons lie within their ensheathing Schwann cells it is difficult to be absolutely certain that GAP-43 immunoreactivity associated with Schwann cells in intact nerve segments does not derive originally from an axonal source, as suggested by Tetzlaff et al. (1989). However, this study has clearly shown using metabolic labeling techniques that Schwann cells can synthesize GAP-43 in vitro. Also, in the chronically denervated distal stump the number of Schwann cells expressing GAP-43 increased in the absence of regenerating axons, suggesting that Schwann cells are capable of

GAP-43 synthesis in vivo. Furthermore, the presence of immunoreactive GAP-43 in membranes isolated from cultured Schwann cells suggests that these cells process GAP-43 in the same manner as neurons, by insertion into the plasmalemma probably after fatty acylation (Skene and Virág, 1989). Our preliminary data on the density dependence of GAP-43 expression in cultured Schwann cells suggest that Schwann cells may themselves secrete factors that regulate GAP-43 expression, but further experiments will be required to clarify the mechanism involved.

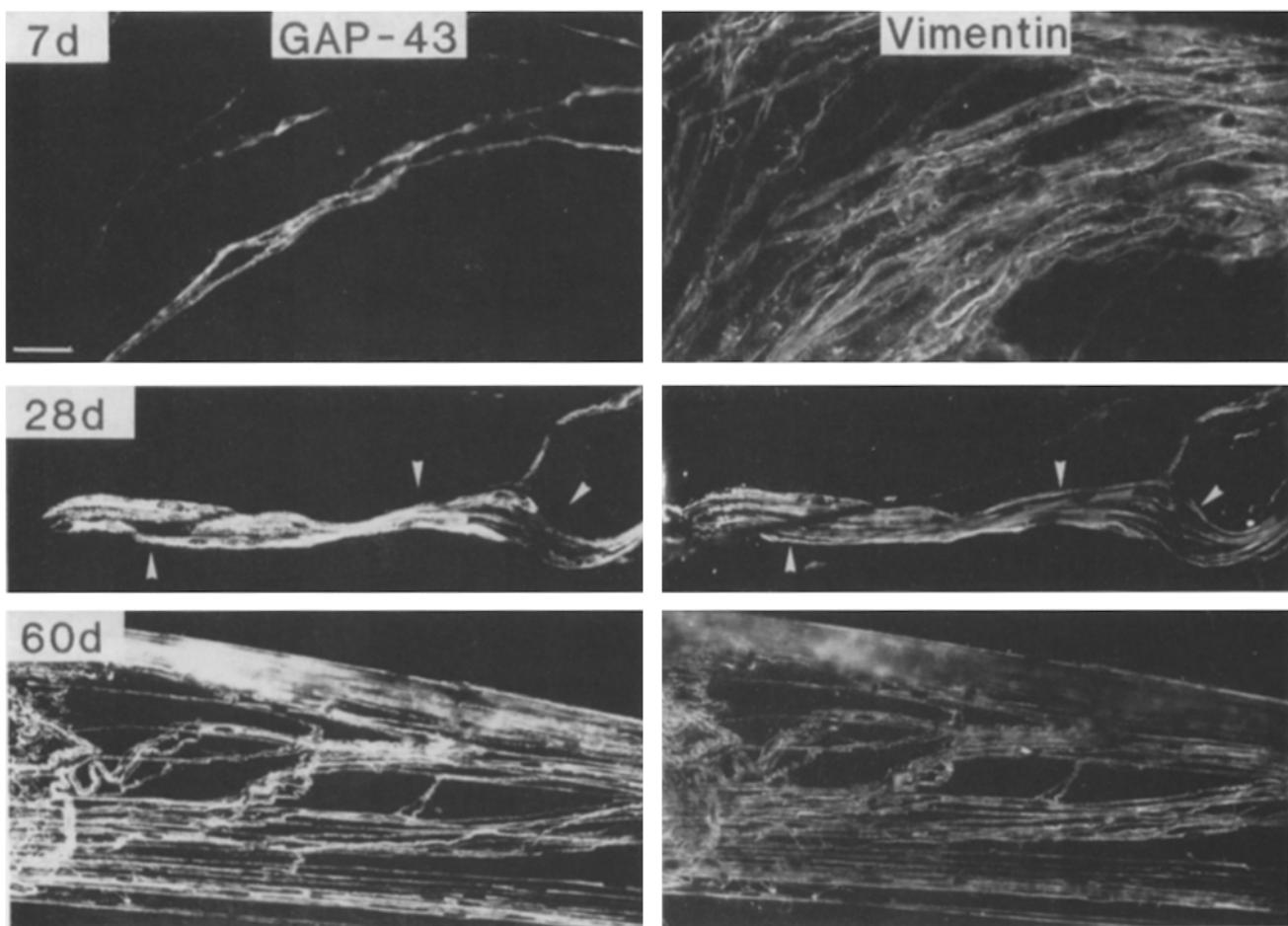
#### **Role Of GAP-43 in Nonmyelin-forming Schwann Cells**

We have previously established that astrocytes of the CNS and nonmyelin-forming Schwann cells express a series of common molecules, including N-CAM, A5E3, and GFAP, suggesting that they may represent a functionally related group of cells (Jessen and Mirsky, 1984). The findings presented in this paper are to some extent consistent with these observations, in that GAP-43 has also been detected in astrocytes in vitro; and we have found that GAP-43 is lost from oligodendrocytes, the CNS equivalents of myelin-forming Schwann cells, early in their development (Curtis et al., 1991). Furthermore, GAP-43 arises early in both these glial cell lineages, being present in oligodendrocyte/type 2 astrocyte progenitors and Schwann cell precursors. In addition, we have previously suggested that GAP-43 may be involved in the motility of oligodendrocyte/type 2 astrocyte progenitors (Curtis et al., 1991) and GAP-43 may serve a similar function in Schwann cell precursors as these are also likely to be highly motile.

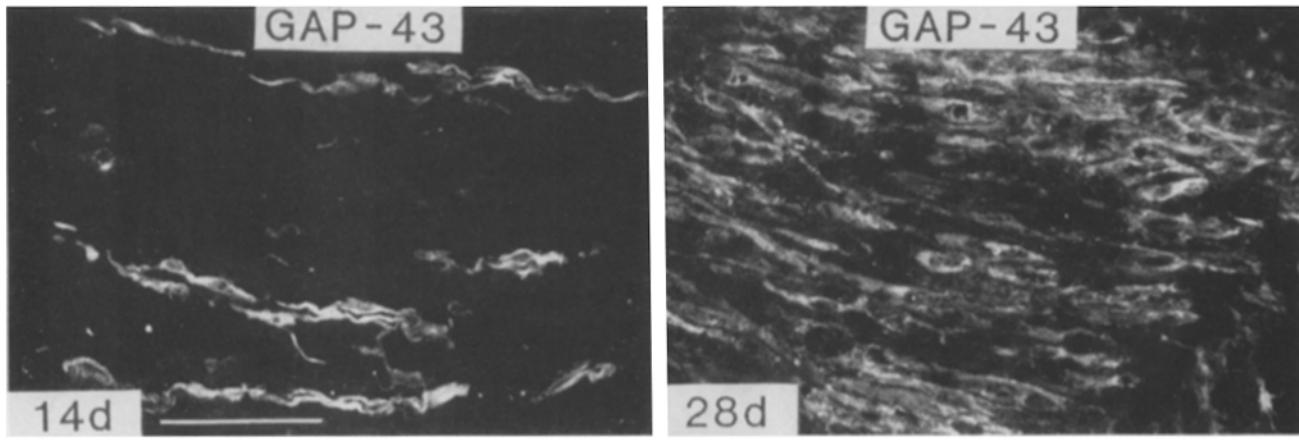
The function of GAP-43 in the nonmyelin-forming Schwann cell is currently unknown. In axonal growth cones, however, GAP-43 is localized in areas of substrate adhesion and associates with the actin-rich membrane cortex (Moss et al., 1990; Meiri and Gordon-Weeks, 1990) and these authors have suggested a role for GAP-43 in cell shape changes induced by alterations in the membrane skeleton. Unmyelinated axons have been shown to undergo spatial and temporal changes in diameter due to the transport of membranous organelles (Greenberg et al., 1990). Nonmyelin-forming Schwann cells ensheathe axons in membrane-bound troughs, and may be required to change shape in response to such variations in axonal caliber. Moreover, junctions between adjacent nonmyelin-forming Schwann cells have not been detected (Aguayo et al., 1976b) and there are no reports of junctions between these cells and their axons. In contrast, myelin lamellae are secured together by tight junctions and myelin-forming Schwann cells are firmly adherent to the axolemma at the nodes of Ranvier (Bunge et al., 1967; Livingston et al., 1973; Mugnaini and Schnapp, 1974; Shinowara et al., 1980). Together, these observations suggest that nonmyelin-forming Schwann cells have a more "plastic" morphology than myelin-forming cells and may undergo changes in shape more readily, features which might involve the interaction of GAP-43 with both plasmalemma and cytoskeleton. This is consistent with the proposed role for GAP-43 in the axonal growth cone.

In conclusion these studies show that Schwann cells synthesize GAP-43 and that GAP-43 immunoreactivity is widespread in Schwann cells in the developing nerve but is restricted to nonmyelin-forming Schwann cells in the mature nerve. Furthermore, loss of axonal contact in vivo leads to

## Teased nerves



## Frozen sections



**Figure 7.** GAP-43 is upregulated in all Schwann cells 4–8 wk after sciatic nerve transection. (*Top*) Teased preparations from denervated distal segments of sciatic nerves were double-labeled with anti-GAP-43 and mAb against vimentin. At 7 d after transection, only a minority of vimentin<sup>+</sup> Schwann cells express GAP-43. At 28 d almost all Schwann cells contain GAP-43, although a few cells remain GAP-43<sup>-</sup> (arrowheads). By 60 d, all Schwann cells visualized with vimentin also contain GAP-43. (*Bottom*) Frozen sections of distal segments after sciatic nerve transection stained with anti-GAP-43. Up to 14 d after transection, GAP-43 is only expressed by a few Schwann cells, which may represent the original population of nonmyelin-forming cells. By 28 d, GAP-43 is widespread in Schwann cells of the distal segment. Bar, 50  $\mu$ m.

upregulation of GAP-43 expression in the progeny of Schwann cells which were previously myelin-forming. These observations may prove to be important in elucidating the function of GAP-43 in the nervous system.

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## References

- Aguayo, A. J., J. Epps, L. Charron, and G. M. Bray. 1976a. Multipotentiality of Schwann cells in cross-anastomosed and grafted myelinated and unmyelinated nerves: quantitative microscopy and radioautography. *Brain Res.* 104:1-20.
- Aguayo, A. J., G. M. Bray, L. C. Terry, and E. Sweezy. 1976b. Three dimensional analysis of unmyelinated fibres in normal and pathologic autonomic nerves. *J. Neuropathol. Exp. Neurol.* 35:136-151.
- Anderton, B. H., D. Breinburg, M. J. Downes, P. J. Green, B. E. Tomlinson, J. Ulrich, J. N. Wood, and J. Kahn. 1982. Monoclonal antibodies show that neurofibrillary tangles and neurofilaments share antigenic determinants. *Nature (Lond.)*. 298:84-86.
- Andreasen, T. J., C. W. Leutje, W. Heideman, and D. R. Storm. 1983. Purification of a novel calmodulin binding protein from bovine cerebral cortex membranes. *Biochemistry*. 22:4615-4618.
- Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman. 1984. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of *N*-acetylglucosamine. *Cell*. 39:405-416.
- Benowitz, L. I., and A. Routtenberg. 1987. A membrane phosphoprotein associated with neural development, axonal regeneration, phospholipid metabolism, and synaptic plasticity. *Trends Neurosci.* 10:527-532.
- Brockes, J. P., K. L. Fields, and M. C. Raff. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* 165:105-118.
- Bunge, M. B., R. P. Bunge, E. R. Peterson, and M. R. Murray. 1967. A light and electron microscope study of long-term organized cultures of rat dorsal root ganglia. *J. Cell Biol.* 32:439-466.
- Chan, S. Y., K. Murakami, and A. Routtenberg. 1986. Phosphoprotein F1: purification and characterization of a brain kinase C substrate related to plasticity. *J. Neurosci.* 6:3618-3627.
- Curtis, R., R. Hardy, R. Reynolds, B. A. Spruce, and G. P. Wilkin. 1991. Down-regulation of GAP-43 during oligodendrocyte development and lack of expression by astrocytes *in vivo*: implications for macroglial differentiation. *Eur. J. Neurosci.* 3:876-886.
- da Cunha, A., and L. Vitković. 1990. Regulation of immunoreactive GAP-43 expression in rat cortical macroglia is cell type specific. *J. Cell Biol.* 111:209-215.
- Deloulme, J.-C., T. Janet, D. Au, D. R. Storm, M. Sensenbrenner, and J. Baudier. 1990. Neuromodulin (GAP43): a neuronal protein kinase C substrate is also present in O-2A glial cell lineage. Characterization of neuromodulin in secondary cultures of oligodendrocytes and comparison with the neuronal antigen. *J. Cell Biol.* 111:1559-1569.
- Fitzgerald, M., M. L. Reynolds, and L. I. Benowitz. 1991. GAP-43 expression in the developing rat lumbar spinal cord. *Neuroscience*. 41:187-199.
- Gorgels, T. G. M. F. M. Van Lookeren Campagne, A. B. Oestreicher, A. A. M. Gribnau, and W. H. Gispens. 1989. B-50/GAP43 is localized at the cytoplasmic side of the plasma membrane in developing and adult rat pyramidal tract. *J. Neurosci.* 9:3861-3869.
- Goslin, K., D. J. Schreyer, J. H. P. Skene, and G. Bunker. 1988. Development of neuronal polarity: GAP-43 distinguishes axonal from dendritic growth cones. *Nature (Lond.)*. 336:672-674.
- Greenberg, M. M., C. Leitao, J. Trogadis, and J. K. Stevens. 1990. Irregular geometries in normal unmyelinated axons: a 3D serial EM analysis. *J. Neurocytol.* 19:978-988.
- Jacobson, R. D., I. Virág, and J. H. P. Skene. 1986. A protein associated with axon growth, GAP-43, is widely distributed and developmentally regulated in rat CNS. *J. Neurosci.* 6:1843-1855.
- Jessen, K. R., and R. Mirsky. 1984. Nonmyelin-forming Schwann cells coexpress surface proteins and intermediate filaments not found in myelin-forming cells: a study of Ran-2, A5E3 antigen and glial fibrillary acidic protein. *J. Neurocytol.* 13:923-934.
- Jessen, K. R., and R. Mirsky. 1991. Schwann cell precursors and their development. *Glia*. 4:185-194.
- Jessen, K. R., R. Mirsky, and L. Morgan. 1987. Myelinated, but not unmyelinated axons, reversibly down-regulate N-CAM in Schwann cells. *J. Neurocytol.* 16:681-688.
- Jessen, K. R., L. Morgan, H. J. S. Stewart, and R. Mirsky. 1990. Three markers of adult non-myelin-forming Schwann cells, 21c(Ran-1), A5E3 and GFAP: development and regulation by neuron-Schwann cell interactions. *Development (Camb.)*. 109:91-103.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Livingston, R. B., K. Pfenninger, H. Moor, and K. Akert. 1973. Specialized paranodal and interparanodal glial-axonal junctions in the peripheral and central nervous system: a freeze-etching study. *Brain Res.* 58:1-23.
- Meiri, K. F., and P. R. Gordon-Weeks. 1990. GAP-43 in growth cones is associated with areas of membrane that are tightly bound to substrate and is a component of a membrane skeleton subcellular fraction. *J. Neurosci.* 10:256-266.
- Meiri, K. F., K. H. Pfenninger, and M. B. Willard. 1986. Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. *Proc. Natl. Acad. Sci. USA*. 83:3537-3541.
- Meiri, K. F., M. Willard, and M. I. Johnson. 1988. Distribution and phosphorylation of the growth-associated protein GAP-43 in regenerating sympathetic neurons in culture. *J. Neurosci.* 8:2571-2581.
- Mirsky, R., and K. R. Jessen. 1991. Schwann cell development and the regulation of myelination. *Semin. Neurosci.* 2:423-439.
- Moss, D. J., P. Fernyhough, K. Chapman, L. Baizer, D. Bray, and T. Allsopp. 1990. Chicken growth-associated protein GAP-43 is tightly bound to the actin-rich neuronal membrane skeleton. *J. Neurochem.* 54:729-736.
- Mugnaini, E., and B. Schnapp. 1974. Possible role of zonula occludens of the myelin sheath in demyelinating conditions. *Nature (Lond.)*. 251:725-727.
- Raible, D. W., and F. A. Morris. 1989. Cyclic AMP regulates the rate of differentiation of oligodendrocytes without changing the lineage commitment of their progenitors. *Dev. Biol.* 133:437-446.
- Shinowara, N. L., W. B. Beutel, and J. -P. Revel. 1980. Comparative analysis of junctions in the myelin sheath of central and peripheral axons of fish, amphibians and mammals: a freeze-fracture study using complementary replicas. *J. Neurocytol.* 9:15-38.
- Skene, J. H. P. 1989. Axonal growth-associated proteins. *Annu. Rev. Neurosci.* 12:127-156.
- Skene, J. H. P., and I. Virág. 1989. Posttranslational membrane attachment and dynamic fatty acylation of a neuronal growth cone protein, GAP-43. *J. Cell Biol.* 108:613-624.
- Skene, J. H. P., and M. Willard. 1981a. Changes in axonally transported proteins during axon regeneration in toad retinal ganglion cells. *J. Cell Biol.* 89:86-95.
- Skene, J. H. P., and M. Willard. 1981b. Axonally transported proteins associated with axon growth in rabbit central and peripheral nervous systems. *J. Cell Biol.* 89:96-103.
- Skene, J. H. P., R. D. Jacobson, G. J. Snipes, C. B. McGuire, J. J. Norden, and J. A. Freeman. 1986. A protein induced during nerve growth (GAP-43) is a major component of growth-cone membranes. *Science (Wash. DC)*. 233:783-786.
- Tanuchi, M., H. B. Clark, and E. M. Johnson. 1986. Induction of nerve growth factor receptor in Schwann cells after axotomy. *Proc. Natl. Acad. Sci. USA*. 83:4094-4098.
- Tetzlaff, W., H. Zwiers, K. Lederis, L. Cassar, and M. A. Bisby. 1989. Axonal transport and localization of B-50/GAP-43-like immunoreactivity in regenerating sciatic and facial nerves of the rat. *J. Neurosci.* 9:1303-1313.
- Verhaagen, J., C. O. M. Van Hooff, P. M. Edwards, P. N. E. De Graan, A. B. Oestreicher, P. Schotman, F. G. I. Jennekens, and W. H. Gispens. 1986. The kinase C substrate protein B-50 and axonal regeneration. *Brain Res. Bull.* 17:737-741.
- Vitković, L., H. W. Steisslinger, V. J. Aloyo, and M. Mersel. 1988. The 43-kDa neuronal growth-associated protein (GAP-43) is present in plasma membranes of rat astrocytes. *Proc. Natl. Acad. Sci. USA*. 85:8296-8300.
- Woolf, C. J., M. L. Reynolds, C. Molander, C. O'Brien, R. M. Lindsay, and L. I. Benowitz. 1990. The growth-associated protein GAP-43 appears in dorsal root ganglion cells and in the dorsal horn of the rat spinal cord following peripheral nerve injury. *Neuroscience*. 34:465-478.