

Schwann Cells of the Myelin-forming Phenotype Express Neurofilament Protein NF-M

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Abstract. Immature Schwann cells of the rat sciatic nerve can differentiate into myelin-forming or non-myelin-forming cells. The factors that influence this divergent development are unknown but certain markers such as galactocerebroside distinguish the two cell populations at an early stage of Schwann cell differentiation. Because myelination requires extensive changes in cell morphology, we have investigated the composition and structure of the Schwann cell cytoskeleton at a time when these cells become committed to myelination. Here we show that Schwann cells express a cytoskeletal protein of *M_r* 145 before diverging into the myelin-forming path, i.e., before they acquire cell-surface galactocerebroside. The p145 protein has the characteristics of an intermediate filament (IF) protein and immunoelectron microscopy shows that it colocalizes

with vimentin, which suggests that these two proteins can coassemble into IFs. Elevated intracellular cAMP levels, which can mimic some of the early effects of axons on Schwann cell differentiation, induced p145 synthesis, therefore, we conclude that myelin-forming Schwann cells express this protein at a very early stage in their development. Immunological comparisons with other IF proteins revealed a close similarity between p145 and the neurofilament protein NF-M; the identification of p145 as NF-M was confirmed by isolating and sequencing a full-length clone from a Schwann cell cDNA library. These data demonstrate that Schwann cells remodel their IFs by expressing NF-M before acquiring the myelin-forming phenotype and that IF proteins of the neurofilament-type are not restricted to neurons in the vertebrate nervous system.

SCHWANN cells differentiate into either a myelin-forming or a non-myelin-forming type during the development of the peripheral nervous system (for a review, see Jessen and Mirsky, 1991). As a result of axonal contact, myelin-forming Schwann cells acquire the differentiation-specific glycolipid galactocerebroside (GalC)¹ around embryonic day 18 (E18) at a time when larger axons segregate into 1:1 units with Schwann cells (Eccleston et al., 1987); non-myelin-forming Schwann cells express GalC much later between postnatal weeks 2–5 (Jessen and Mirsky, 1985; Jessen et al., 1987). Close contact with axons is also necessary for the expression of a second glycolipid, sulfatide, which is identified by the mAb O4 (Sommer and Schachner, 1981; Mirsky et al., 1990); however, unlike GalC, O4 is expressed in both types of Schwann cell in the rat sciatic nerve at an early stage (Mirsky et al., 1990). By E18, Schwann cells also express a distinct set of proteins including A5E3, Ran-1, and glial fibrillary acidic protein (GFAP) (Jessen et al., 1990). In non-myelin-forming Schwann cells, Ran-1, A5E3, and GFAP persist into adulthood whereas all three proteins are down-regulated in myelinating Schwann cells.

Axonal contact stimulates Schwann cell proliferation (Salzer and Bunge, 1980; DeVries et al., 1982; Sobue and Plea-

sure, 1985), basement membrane formation (Bunge et al., 1986), and is also critical for myelin synthesis (Mirsky et al., 1980; Bunge et al., 1986; Lemke and Chao, 1988; Brunden et al., 1990). Some of the results of axonal contact such as Schwann cell proliferation and the cell surface expression of GalC can be mimicked in vitro by cAMP and its analogues (Sobue and Pleasure, 1984, 1986; Porter et al., 1986). Recently, Morgan et al. (1991) showed that the myelin-forming phenotype (up-regulated P₀ and down-regulated Ran-1, A5E3, and GFAP) could be induced in quiescent dissociated Schwann cells by raising intracellular cAMP levels. In contrast, O4 expression was equally inducible in dividing and nondividing cells.

Myelination requires dramatic changes in the shape of the Schwann cell which must involve the cytoskeleton. Indeed the cytoskeleton of oligodendrocytes has already been shown to be intimately involved in the extension of myelin processes in the rat central nervous system (Wilson and Brophy, 1989). In most eukaryotic cells the cytoskeleton consists of microtubules (diameter 25 nm), microfilaments (diameter 5–7 nm), and intermediate filaments (IFs) (diameter 10 nm). IFs are morphologically similar in most eukaryotic cell types but they show a wide heterogeneity in their polypeptide subunits and these have been divided into six classes: types I and II (keratins in epithelial cells), type III (vimentin, desmin, GFAP, peripherin), type IV (the neurofilaments NF-L, NF-M

1. *Abbreviations used in this paper:* GalC, glycolipid galactocerebroside; GFAP, glial fibrillary acidic protein; IF, intermediate filament.

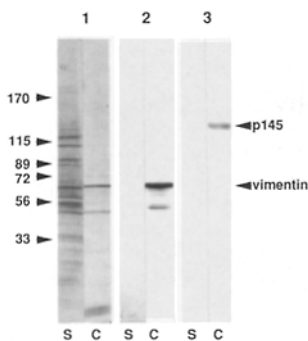


Figure 1. Immunoblot of cytoskeleton (C) and soluble (S) fractions of Schwann cells (80 μ g protein) from 5-d rat sciatic nerves as described in Materials and Methods. (1) Proteins stained with Amido Black; (2) immunoblot with anti-vimentin; (3) immunoblot with anti-p145. Both vimentin and p145 were exclusively associated with the Schwann cell cytoskeleton and their respective antibodies were specific.

and NF-H of apparent molecular masses 68, 150, and 200 kD, respectively, and α -internexin), type V (nuclear lamins), and type VI (nestin) (Lendahl et al., 1990; Steinert and Liem, 1990; Chin et al., 1991). Since IF proteins are often specifically expressed in particular cell types, they have been used as differentiation markers and as such are useful tools in the study of cell differentiation as well as in tumour identification (Osborn and Weber, 1983).

All IF proteins share common structural features: they are all rod-shaped and have a large, highly conserved central α -helical core domain of \sim 310 residues which is flanked by two nonhelical terminal domains of variable length and sequence at the amino- and carboxy-terminal regions (Weber et al., 1983; Monteiro and Cleveland, 1989). Despite extensive knowledge of their amino acid and cDNA sequences, the physiological function of most IF proteins remains elusive. Their linkage to both nucleus and plasma membrane (Georgatos et al., 1987) suggests a general role in the spatial organization of the cytoplasm; alternatively, IFs may be involved either in the transport of macromolecules between the nucleoplasmic and cytoplasmic compartments or in the transduction of information from the cell periphery to the nucleus

(Geiger, 1987; Goldman et al., 1985). Recent transfection experiments have shown that GFAP is required for the formation of stable astrocytic processes in response to neurons (Weinstein et al., 1991), and neurofilaments may determine axonal caliber (Hoffman et al., 1983), although the results of recent transgenic experiments in mice argue to the contrary (Monteiro et al., 1990).

IFs are commonly remodelled during cell maturation which suggests that the IF network has a role in differentiation. Thus, vimentin is exchanged for desmin in the IFs of developing muscle cells (Lazarides, 1982; Cossette and Vincent, 1991), and there are dramatic changes in the composition of IF networks during neuronal development. Neuroepithelial stem cells initially coexpress nestin and vimentin (Lendahl et al., 1990), but nestin is subsequently down-regulated and the type IV IF protein α -internexin appears (Fliegner et al., 1990). α -Internexin is in turn replaced by the type IV proteins NF-L, NF-M, and NF-H that are characteristic of mature neurons (Steinert and Liem, 1990). The phenomenon of altered IF protein expression during terminal differentiation is also evident in glia. Mature oligodendrocytes lack IF proteins but their progenitors possess vimentin (Noble et al., 1990). Similarly, vimentin is the IF protein of immature Schwann cells although, unlike oligodendrocytes, vimentin is retained in differentiated cells (Autilio-Gambetti et al., 1982). A second IF protein, GFAP, appears in Schwann cells at E18 (Jessen and Mirsky, 1991) but the expression of GFAP is suppressed upon terminal differentiation of myelin-forming Schwann cells, while it is retained in non-myelin-forming Schwann cells (Jessen et al., 1990).

We are interested in the role of the cytoskeleton in the differentiation of myelin-forming cells and in how the cytoskeleton regulates the extension of myelin processes during axonal ensheathment. In this paper we describe a Schwann cell IF protein that is expressed at an early stage in the differentiation program of myelin-forming Schwann cells. This protein, which we initially called p145, colocalizes with

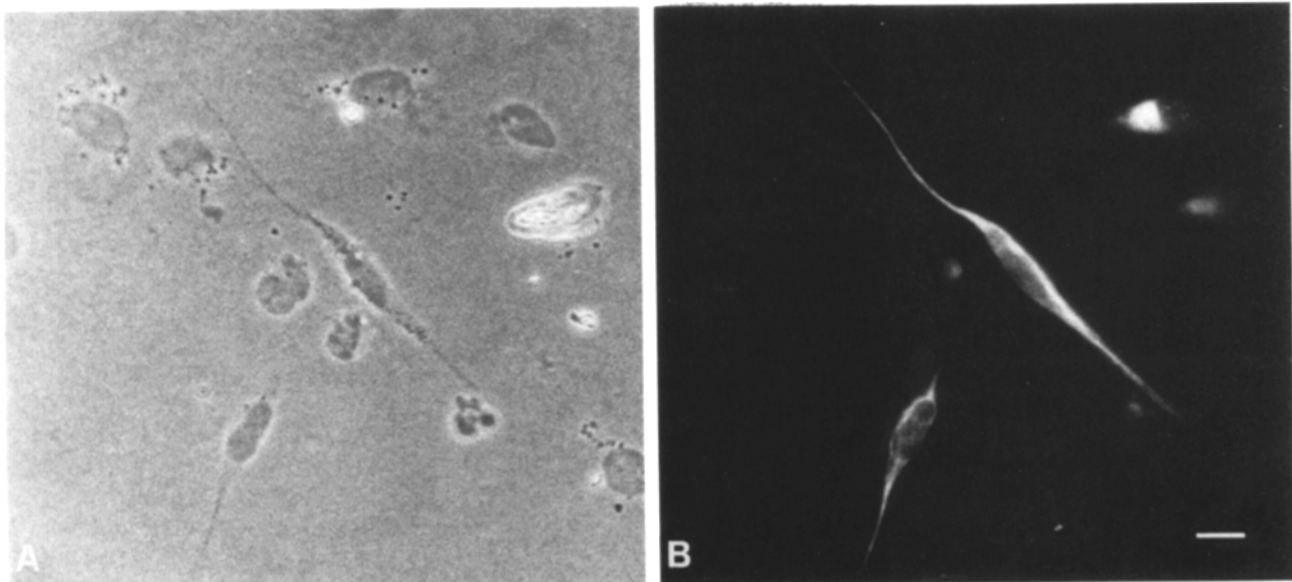


Figure 2. Immunofluorescence localization of p145 in Schwann cell cytoskeletons. Cells were grown on poly-D-lysine-coated plastic for 24 h before extraction. Cytoskeletons were viewed with (A) phase-contrast optics and (B) rhodamine optics to visualize p145. Note the filamentous staining in the lower cell. Bar, 10 μ m.

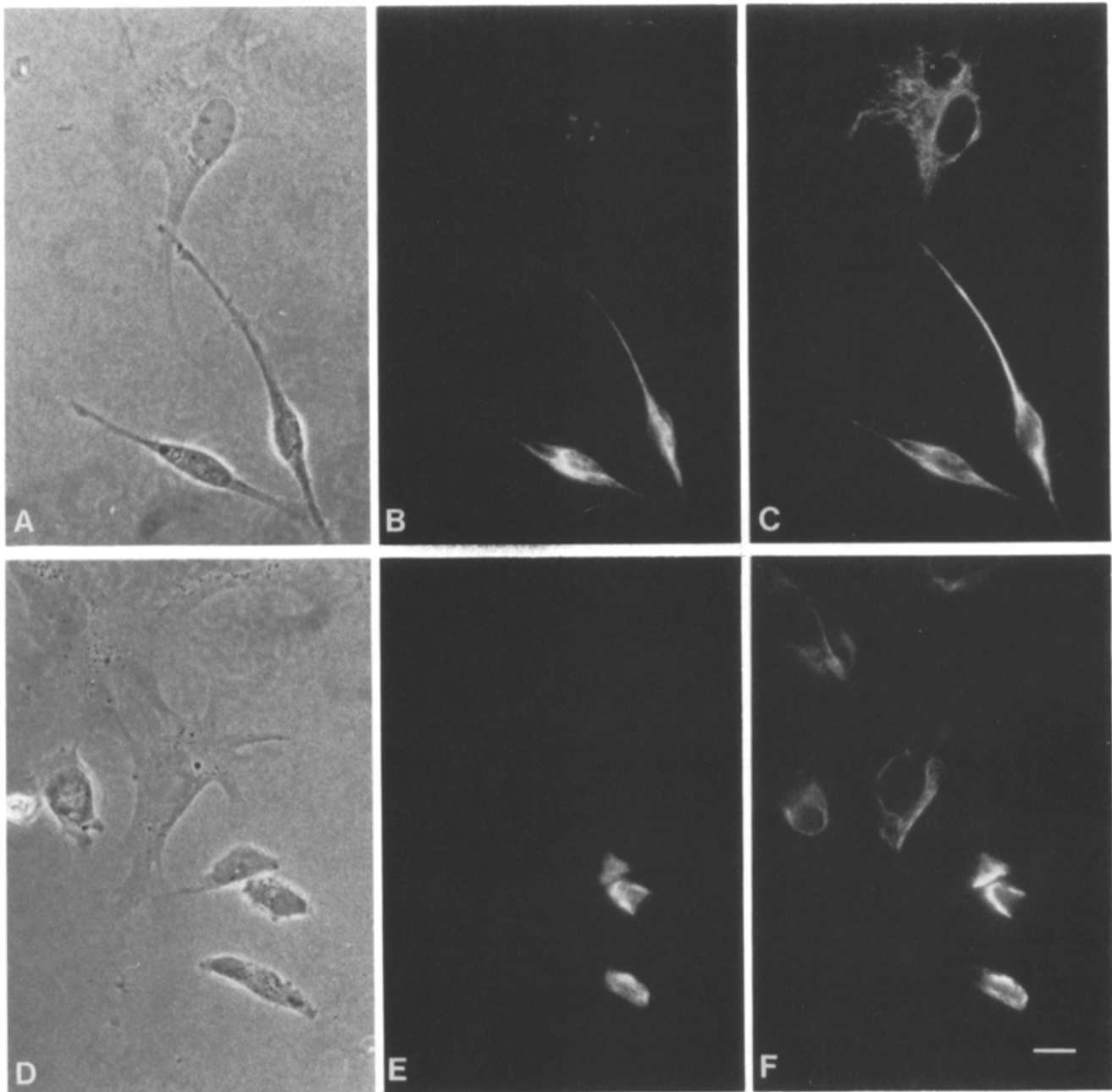


Figure 3. Double-label immunofluorescence localization of p145 and vimentin in Schwann cell cultures treated with colchicine. Cells were viewed before colchicine by (A) phase contrast, (B) rhodamine optics for p145, and (C) fluorescein optics for vimentin. Note that the fibroblast has vimentin but does not contain p145. Colchicine-treated cells are shown by (D) phase-contrast optics, (E) rhodamine optics for p145, and (F) fluorescein optics for vimentin. Both p145 and vimentin have retracted to a perinuclear location. Bar, 10 μm .

vimentin and is inducible in culture by cAMP analogues. Immunological analysis suggested that p145 might be identical to the neurofilament protein NF-M, and this hypothesis was confirmed by cDNA cloning.

Materials and Methods

Schwann Cell Cultures

Sciatic nerves from 1-5-d-old embryonic rats and pups were dissected and dissociated according to the method of Brookes et al. (1979) and the cells were seeded on either 13-mm glass coverslips (BDH, Glasgow, Scotland) or

plastic 35-mm dishes (Costar Data Packaging Corp., Cambridge, MA) that had been coated with 100 $\mu\text{g/ml}$ poly-D-lysine (Sigma Chemical Co., St. Louis, MO). Cultures were maintained in Optimem (Gibco-BRL, Grand Island, NY) containing 10% FCS (Flow Laboratories, Inc., McLean, VA).

Cell Culture in the Presence of cAMP Analogues

To examine the effects of raised intracellular cAMP levels in Schwann cells, cultures from sciatic nerves of 5-d rat pups were grown for 24 h in Optimem containing 10% FCS. The cells were rinsed three times with Optimem and then refed in medium containing 5×10^{-4} M $\text{N}^6, 2'-O$ -dibutyryl adenosine 3':5'-cyclic monophosphate (db-cAMP) (Sigma Chemical Co.) for 48 h in the presence or absence of FCS with media changes every 24 h. Control cultures were refed with fresh Optimem containing 10% FCS every 24 h.

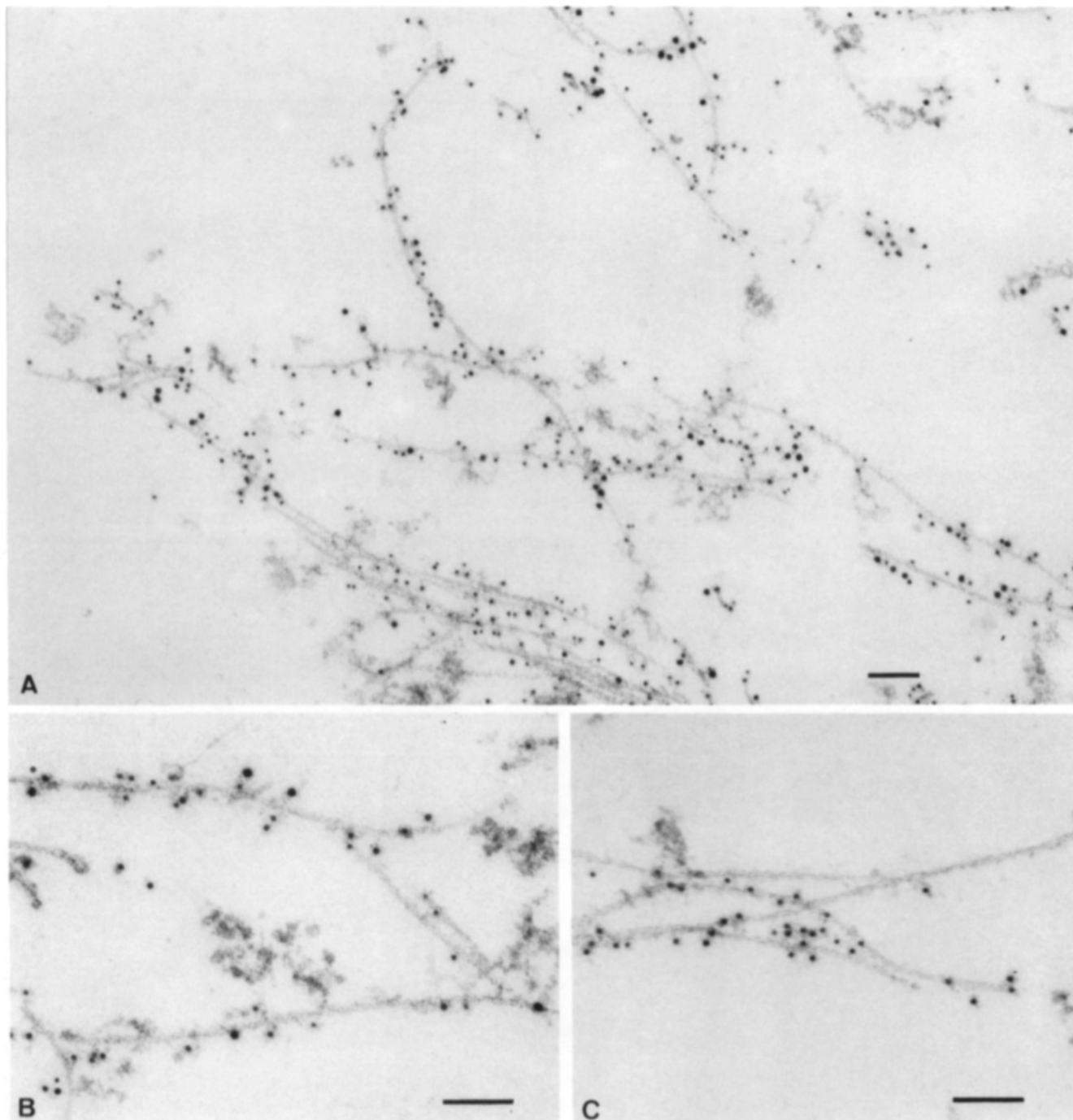


Figure 4. Immunoelectron localization of p145 and vimentin in Schwann cells. Cytoskeletons were immunostained with p145 antibody and vimentin mAb and then reacted with secondary antibodies that were labeled with colloidal gold particles. IFs were clearly immunostained for both p145 (10 nm gold) and vimentin (5 nm gold) (*A* and *B*). Schwann cell IFs composed only of vimentin which were not labeled with anti-p145 are shown in *C*. Bars, 0.1 μm .

In experiments designed to induce expression of P_0 , we used a slight modification of the protocol of Morgan et al. (1991). Briefly, after cell culture for 24 h in Optimem containing 10% FCS, 10^{-5} M cytosine arabinoside (Sigma Chemical Co.) was added for 48 h after which time cultures were returned to Optimem containing 10% FCS for a further 24 h. On the fifth day, cAMP analogues db-cAMP and 8-bromoadenosine-cAMP (Sigma Chemical Co.) each at 5×10^{-4} M were added to Optimem containing 2% FCS. On the sixth day, medium was replenished, but the concentration of each cAMP analogue was reduced to 5×10^{-5} M. After a further 24 h, the medium was replaced by serum-free Optimem containing the cAMP analogues. Finally, on the eighth day of culture, cells were processed for in-

direct immunofluorescence. Control cultures were maintained throughout in Optimem containing 10% FCS and were refed every 24 h.

Preparation of Cytoskeleton Fractions from Schwann Cells

Cell cultures were extracted at room temperature for 3 min with the extraction buffer described by Wilson and Brophy (1989) (10 mM Pipes, pH 6.9, 50 mM KCl, 2 mM EGTA, 1 mM MgCl_2 , 2 M glycerol, 10 $\mu\text{g/ml}$ leupeptin (Sigma Chemical Co.), and 10 $\mu\text{g/ml}$ antipain (Sigma Chemical Co.), containing 0.5% (vol/vol) Triton X-100). The cytoskeletons were then

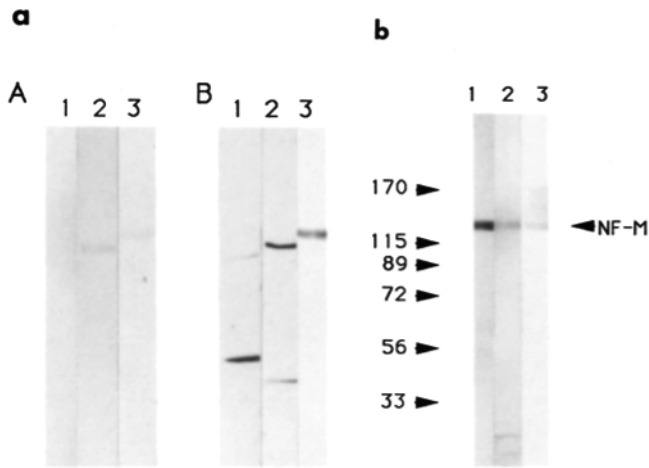


Figure 5. (a) Lysates from *E. coli* (C600) infected with λ gt11 (lane 1) or recombinant λ gt11 containing SNPI01I isolated from a rat sciatic nerve library (lane 2) and Schwann cells (lane 3) were electrophoresed on 5–17% polyacrylamide gels and transferred to nitrocellulose. An antibody (AII) was raised against the β -galactosidase fusion protein that had been expressed in the recombinant cells. Immunoblotting showed that, as expected, Schwann cell p145 was recognized by both anti-p145 (A) and AII (B) confirming that p145 contained NF-M sequences. The low M_r bands detected by AII in lanes 1 and 2 are presumably proteolytic breakdown products. (b) NF-M is immunologically related to p145. Schwann cells from 5-d rat sciatic nerve were cultured for 16 h, solubilized in SDS-PAGE sample buffer, and analyzed by immunoblotting. Both AII (lane 2) and Dahl's rabbit anti-NF-triplet antibody (lane 3) recognize a protein with the same M_r as p145, which is shown in lane 1 stained with anti-p145.

washed in the same buffer without Triton X-100 before being fixed and processed for indirect immunofluorescence. For Western blotting, extract and wash were pooled and centrifuged at 14,000 g for 10 min after which the supernatant was designated the soluble fraction. Cytoskeletons were scraped off with a rubber policeman and sedimented by centrifugation at 14,000 g for 10 min.

Antibodies

Gifts of the following antibodies are gratefully acknowledged. They were used at the indicated dilutions for immunofluorescence and they were diluted a further 10-fold for immunoblotting: rabbit anti-S100 (J. E. Hesketh, Rowett Research Institute, Aberdeen, Scotland) (1:5,000); rabbit antivimentin (R. O. Hynes, M.I.T., Cambridge, MA) (1:200); rabbit anti-P₀ (D. R. Colman, Columbia University, New York) (1:200); affinity-purified 192-IgG mAb against rat nerve growth factor receptor which also recognizes the Ran-1 antigen (Yan and Johnson, 1988; Jessen et al., 1990) (E. M. Johnson, Washington University School of Medicine, St. Louis, MO) (1:100); 01 which is specific for galactocerebroside (GalC) (Bansal et al., 1989) and O4 mAbs (I. Sommer, Southern General Hospital, Glasgow) (1:20 and 1:10, respectively); a mAb raised against GalC that we hereafter refer to as R-mAb whose specificity also overlaps with that of O4 (B. Ranscht, Cancer Research Centre, La Jolla, CA) (1:100). The following antibodies were purchased: NF-L, NF-M, and NF-H mAbs (Sigma Chemical Co.) were each used at a dilution of 1:200; NF-M mAb (Biomakor, Israel) was used at 1:20; NF-L (Boehringer Mannheim Biochemicals, Indianapolis, IN) was used at 1:100; GFAP mAbs from Amersham Corp. (Arlington Heights, IL) and Boehringer Mannheim Biochemicals (clone G-A-5), were both used at 1:5; rabbit anti-GFAPs were either from Dakopatts (Copenhagen) or were a gift from E. Bock (University of Copenhagen) and were used at 1:200; vimentin mAb was from Serotec (Kidlington, Oxford, England) and was used at 1:50. An anti-p145 rabbit antibody was raised against a detergent-insoluble fraction of rat CNS myelin, affinity purified by chromatography on a column of p145 coupled to CNBr-activated Sepharose (Sigma Chemical Co.) and was used at 1:5. The p145 ligand was purified by electroelution from polyacrylamide gels after SDS-PAGE of

PNS myelin (Hunkapiller et al., 1984). The affinity-purified p145 antibody was used at 1:5. A rabbit antibody that recognizes NF-L, NF-M, and NF-H was a gift from D. Dahl (Harvard University Medical School).

Immunoprecipitation

Schwann cells from the sciatic nerves of 5-d-old rat pups were grown for 24 h in Optimem containing 10% FCS, rinsed four times in Optimem, and labeled in Optimem containing 10^{-4} M db-cAMP and [35 S]methionine (1 mCi/ml) (ICN Biochemicals, Buckinghamshire, England) for 24 h at 37°C. After labeling, cells were rinsed extensively with Optimem before extraction with the buffer containing Triton X-100. Cytoskeleton fractions were solubilized in 2% SDS with 1 mM DTT and then diluted with 4 vol of solution A (50 mM Tris-HCl, pH 7.4, 2.5% [vol/vol] Triton X-100, 150 mM NaCl, 4 mM EDTA containing 1 mM Na-p-Tosyl-L-lysine chloromethyl ketone [Sigma Chemical Co.], and 10 μ g/ml leupeptin [Sigma Chemical Co.]). The solubilized samples were precleared with protein A agarose (Sigma Chemical Co.) in solution A. Each sample containing 0.8×10^6 of TCA-precipitable cpm was incubated with a nonimmune serum (2 μ l), affinity-purified p145 antibody (150 μ l), or rabbit anti-bovine vimentin (2 μ l) for 90 min at room temperature followed by protein A agarose for 60 min. Immune complexes were centrifuged at 13,000 g for 5 min and washed six times with solution A containing 0.1% SDS and finally with solution A without detergents. The samples were solubilized and electrophoresed on 5–17% gradient polyacrylamide gels using the buffer system of Laemmli (1970). Gels were stained with 0.2% PAGE Blue 83 (BDH), destained, incubated for 30 min in Amplify (Amersham Corp., Buckinghamshire, England), and dried. The dried gel was exposed to X-ray film (Konica, Stirling, Scotland) at -70°C and the autoradiograph was developed using Kodak X-ray developer and fixer solutions.

Western Blot Analysis

Cytoskeleton pellets and soluble fractions were electrophoresed on 5–17% gradient polyacrylamide gels using the buffer system of Laemmli (1970). The separated proteins were then electro-transferred to nitrocellulose according to the method of Towbin et al. (1979). The nitrocellulose was blocked by incubation for 3 h with 0.2% (wt/vol) gelatin in PBS containing 0.1% (vol/vol) Triton X-100. Proteins were detected by incubation with appropriately diluted primary antibodies in blocking buffer followed by goat anti-rabbit IgG-HRP conjugate (North East Biochemicals, Uxbridge, England). After washing three times with blocking buffer and once with PBS, immunoreactive proteins were detected using 3,3'-DAB HCl and H₂O₂ (Sigma Chemical Co.) as substrates in 50 mM Tris-HCl, pH 7.4. The peroxidase reaction was terminated with 2% SDS.

Indirect Immunofluorescence

When used on living cells, antibodies were diluted in PBS containing 2% goat serum (Scottish Antibody Production Unit, Carlisle, Lanarkshire, Scotland) before fixation. Otherwise, cells were first fixed in 3.7% (wt/vol) paraformaldehyde (Taab) and quenched with 0.1 M glycine in PBS. To detect intracellular antigens, fixed cells were permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 5 min. For GFAP immunocytochemistry, cells were fixed in ethanol-acetic acid (95:5) for 10 min at -20°C . Fixed cells were blocked with 0.2% (wt/vol) gelatin in PBS. Appropriately diluted antibodies in PBS/gelatin were incubated for 60 min with permeabilized cells and fixed cytoskeletons. The cells were washed and then stained for 30 min with fluorescein-labeled anti-mouse and rhodamine-labeled anti-rabbit Ig (North East Biochemical Laboratories, Uxbridge, England) each diluted 50-fold with blocking buffer containing 10% goat serum. Coverslips were washed several times with blocking buffer and finally with PBS and then mounted on glass slides using 90 (vol/vol) glycerol, 10% (vol/vol) PBS, 0.1% (wt/vol) 1,4-phenylenediamine (Aldrich Chemical Company, Dorset, England). Fluorescence was viewed using 40 \times or 63 \times objectives on a Zeiss microscope equipped with phase-contrast and epifluorescence optics. Kodak T-Max (400 ASA) film was used for all photomicrography. All quoted errors in cell counts are SEMs.

Electron Microscopy

Schwann cells cultured on aclar discs (Allied Chem. Corp., Waltham, MA) were extracted to yield cytoskeletons which were then fixed for 30 min in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. After quenching in 100 mM ammonium chloride, nonspecific protein binding sites on cytoskeletons were blocked with 0.3% (wt/vol) BSA, 0.5% (vol/vol) fish

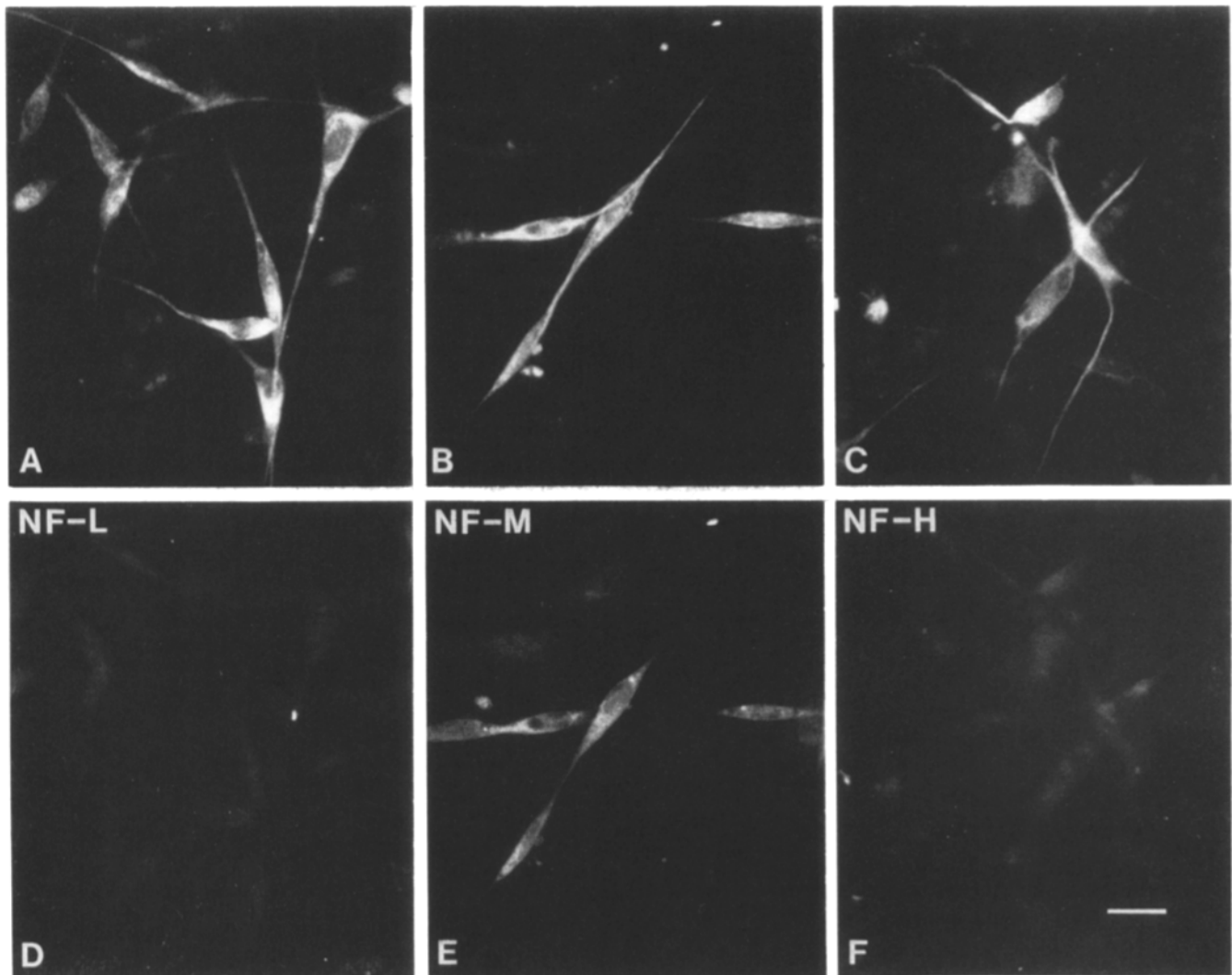


Figure 6. Double-label immunofluorescence of cells with anti-p145 and NF mABS. Cells were viewed with rhodamine optics to visualize p145 (A–C) and fluorescein optics to visualize NF-L (D), NF-M (E), and NF-H (F). Only NF-M mAb stained Schwann cells and colocalized with p145 (B and E). Bar, 20 μ m.

gelatin (Sigma Chemical Co.), in 20 mM Tris containing 200 mM NaCl, pH 7.4. Primary antibodies were diluted with blocking buffer and incubated for 2 h with cytoskeletons in a humid chamber at room temperature. After washing with blocking buffer, incubations with gold-labeled secondary antibodies were carried out at room temperature for 2 h in a humid chamber. The goat anti-rabbit IgG-10-nm gold (Janssen, Olen, Belgium) and 5-nm gold-labeled goat anti-mouse IgG + 1 gM (H + L) (Amersham Corp.) antibodies were each diluted 20-fold in blocking buffer. After washes in Tris/NaCl and 0.1 M sodium cacodylate buffer, pH 7.4, samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate for 30 min at room temperature and left overnight in the same buffer. Samples were postfixated with 1% OsO₄ in 1% potassium ferrocyanide in 0.1 M sodium cacodylate for 60 min at 4°C and then dehydrated in a graded ethanol series: 50%-5 min, 70%-5 min, 90%-5 min, absolute-10 min (twice), absolute-5 min. The infiltration protocol was Epon (Taab)-ethanol (1:1) for 1 h at room temperature, Epon, two changes during the next 12 h followed by embedding in fresh Epon and polymerization at 60°C for 12–24 h. Pale-gold sections were cut on an LKB III ultra microtome and sections were viewed on a Jeol 100 CX transmission electron microscope.

Isolation and Characterization of cDNA clones from Rat Sciatic Nerve and Schwann Cell cDNA Libraries

Total RNA was extracted from P10 rat sciatic nerve and from cAMP-induced P4 rat Schwann cells with RNazol B (Biogenesis, Bournemouth, England) following the manufacturer's instructions, following which poly-

(A)⁺ RNA was isolated (Aviv and Leder, 1972). For the sciatic nerve cDNA library, first and second strand synthesis was carried out by standard methods (Maniatis et al., 1989). After size fractionation on a Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, NJ) column, EcoRI-linked cDNA was ligated to λ gt11 arms (Promega Biotec, Madison, WI) and packaged using Gigapack Gold (Stratagene, Cambridge, England). The Schwann cell library was constructed unidirectionally in SalI/NotI double-digested λ gt22A (Gibco BRL) following the manufacturer's instructions. Affinity-purified anti-p145 antibodies were used to screen 5.0×10^5 and 2.5×10^5 recombinants in the sciatic nerve and Schwann cell libraries, respectively (Young and Davis, 1983), and positive clones were identified using a goat antirabbit antibody conjugated to HRP (North East Laboratories). λ DNAs were isolated from plaque-purified clones (Lockett, 1990) and the excised cDNA inserts were subcloned into pIBI30 (IBI) or pSPORT 1 (Gibco BRL) for further analysis.

Subcloned cDNA inserts (1 ng) or the cDNA products from reverse transcribing total RNA (5 μ g) from cAMP-induced P4 rat Schwann cells using oligo(dT) as primer (Doherty et al., 1989) were amplified by PCR with NF-M-specific primers. Each reaction was subjected to 30 cycles of amplification with 2.5 U Ampli-Taq in a total volume of 100 μ l of a buffer supplied by the manufacturer (Perkin-Elmer Cetus, Norwalk, CT). The primers used for amplification spanned a 1,234-bp segment of the NF-M sequence between bases 1288 and 2522 using the numbering system of Napolitano et al. (1987).

Restriction endonuclease fragments directionally subcloned into M13 mpl8 and mpl9 vectors (New England Biolabs, CP Laboratories, Hertford-

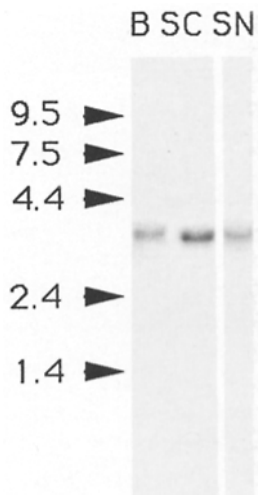


Figure 7. Total RNA was prepared from rat brain (B), P4 Schwann cells that had been induced with db-cAMP (SC) and P3 sciatic nerve (SN). RNA (5, 5, and 15 μ g, respectively) was electrophoresed on a 1.2% formaldehyde agarose gel and transferred to Hybond N membrane. The filters were probed with a Bst EII/DraI fragment of the II cDNA that was 32 P labeled by random priming. The autoradiograph shows that the probe recognized a mRNA of \sim 3 kb in B, SC, and SN thereby supporting the identity of p145 with NF-M.

shire, England) and unidirectional deletions (Henikoff, 1984) prepared in the Phagescript vector (Stratagene) were sequenced by the dideoxy chain termination technique (Sanger et al., 1977) using Sequenase (United States Biochemical Corp., Cleveland, OH).

Fusion Protein and Antifusion Protein Antibodies

E. coli (strain C600) was infected with either λ gt11 or a recombinant λ gt11 containing a p145 clone pSNP10II isolated from the sciatic nerve cDNA library, whose sequence was identical to that of rat NF-M between nucleotides 2137 and 2545, numbered according to Napolitano et al. (1987). The fusion protein was isolated by preparative SDS-gel electrophoresis of *E. coli* lysates and an antiserum (AII) was produced in rabbits. Lysates from cells infected with either nonrecombinant λ gt11 or recombinant λ gt11 containing the pSNP10II clone were electrophoretically resolved, transferred to nitrocellulose, and immunoblotted with AII and affinity-purified anti-p145 at dilutions of 1:1,000 and 1:50, respectively.

Northern Blot Analysis

Total RNA (5–15 μ g) from rat sciatic nerve, cAMP-induced Schwann cells, and brain was electrophoresed on 1.2% formaldehyde agarose gels (Kroczek and Siebert, 1990) and transferred to Hybond N membrane (Amersham Corp.) by vacuum blotting in $20 \times$ SSC. The filters were probed for 2 h in QuickHyb solution (Stratagene) with a BstEII/DraI fragment of the pSNP10II clone that was 32 P-labeled by random priming (Feinberg and Vogelstein, 1983) and they were washed to a final stringency of $0.2 \times$ SSC at 65°C .

Results

p145 Is a Schwann Cell Cytoskeleton Protein

Schwann cells were isolated from the sciatic nerves of 5-d-old rat pups and cultured for 24 h in Optimem containing 10% FCS. Cells were then extracted with an extraction buffer containing Triton X-100 (Wilson and Brophy, 1989); the resultant cytoskeleton and soluble fractions were electrophoresed on SDS-PAGE and the separated proteins were analyzed by Western blotting. Immunoblotting with affinity-purified anti-p145 and rabbit antivimentin demonstrated that, like vimentin, all of the p145 remains associated with the cytoskeleton fraction and that the anti-p145 specifically recognizes only p145 in Schwann cell cytoskeletons; the vimentin antibody was similarly specific (Fig. 1). The presence of p145 in Schwann cell cytoskeletons was also demonstrated by indirect immunofluorescence (Fig. 2).

p145 Is an IF Protein

The Schwann cell cultures used in some of these experiments

also contained fibroblasts. Schwann cells were identified by the phenotype S100⁺, Thy 1⁻, fibroblasts by the phenotype S100⁻, Thy 1⁺, and expressors of p145 were always Thy 1⁻. The staining pattern of p145 by immunofluorescence in Schwann cells was intracellular and filamentous and was very similar to that observed for vimentin (Fig. 3, B and C). It has long been recognized that when cultured cells are treated with cytoskeleton-disrupting drugs such as colchicine, the classic response of IF proteins is to retract from the cell periphery and reorganize around the nucleus (Yang et al., 1985). This perinuclear bundling of IFs was observed for both p145 and vimentin in response to colchicine treatment (Fig. 3, E and F). When colchicine-treated cultures were allowed to recover in medium without colchicine for a further 24 h, both p145 and vimentin expression reverted to the normal staining pattern (data not shown). The cytoskeleton association and response of p145 to colchicine treatment indicated that this protein may be part of the IF network. Further proof for this hypothesis was provided by immunoelectron microscopy. Schwann cell cytoskeletons were prepared and double labeled with anti-p145 and vimentin mAb followed by gold-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies (10 and 5 nm, respectively). The two different sized gold particles decorated the same 10-nm filaments indicating that p145 is a component of IFs and that p145 and vimentin reside on the same IFs (Fig. 4 A). The coresidence of p145 and vimentin is more clearly visible at higher magnification (Fig. 4 B). In addition to containing IFs composed of both p145 and vimentin, some Schwann cells contained only vimentin IFs and these were not labeled with p145 (Fig. 4 C).

cDNAs from Sciatic Nerve and Schwann Cell Libraries Show p145 Is Identical to NF-M

We have provided evidence that p145 is an IF protein of Schwann cells; clearly the relationship of this protein to other well-characterized IF proteins is an important question. To establish the identity of p145, a partial cDNA clone, pSNP10II, was isolated by immune-screening a sciatic nerve cDNA library with anti-p145 and was found to include the sequence between bp 2137–2545 of the rat neurofilament protein NF-M cDNA (Napolitano et al., 1987). An antibody (AII) raised against the β -galactosidase fusion protein product of this clone recognized p145 thus establishing the relationship between p145, pSNP10II, and NF-M (Fig. 5 a). Western blotting showed that both AII and a polyclonal antibody that recognizes the NF triplet NF-L, NF-M, and NF-H cross-reacted with Schwann cell p145 (Fig. 5 b). The absence of NF-L and NF-H from Schwann cells was confirmed by immunofluorescence microscopy (Fig. 6). The NF-M mAb stained Schwann cells in an identical fashion to p145 antibody thus supporting the relatedness of p145 and NF-M (Fig. 6).

Further support for the identity of p145 with NF-M came from Northern blotting in which a mRNA of the same size (\sim 3,000 nucleotides) as that of brain NF-M was detected in sciatic nerve and Schwann cells using pSNP10II as probe (Fig. 7).

To establish the precise relationship between p145 and NF-M we prepared a cDNA library from cAMP-induced Schwann cells, screened it with anti-p145 and isolated 24 positive clones. Seven clones were amplified by PCR with primers that span bp 1288–1310 and 2505–2522 of the NF-M

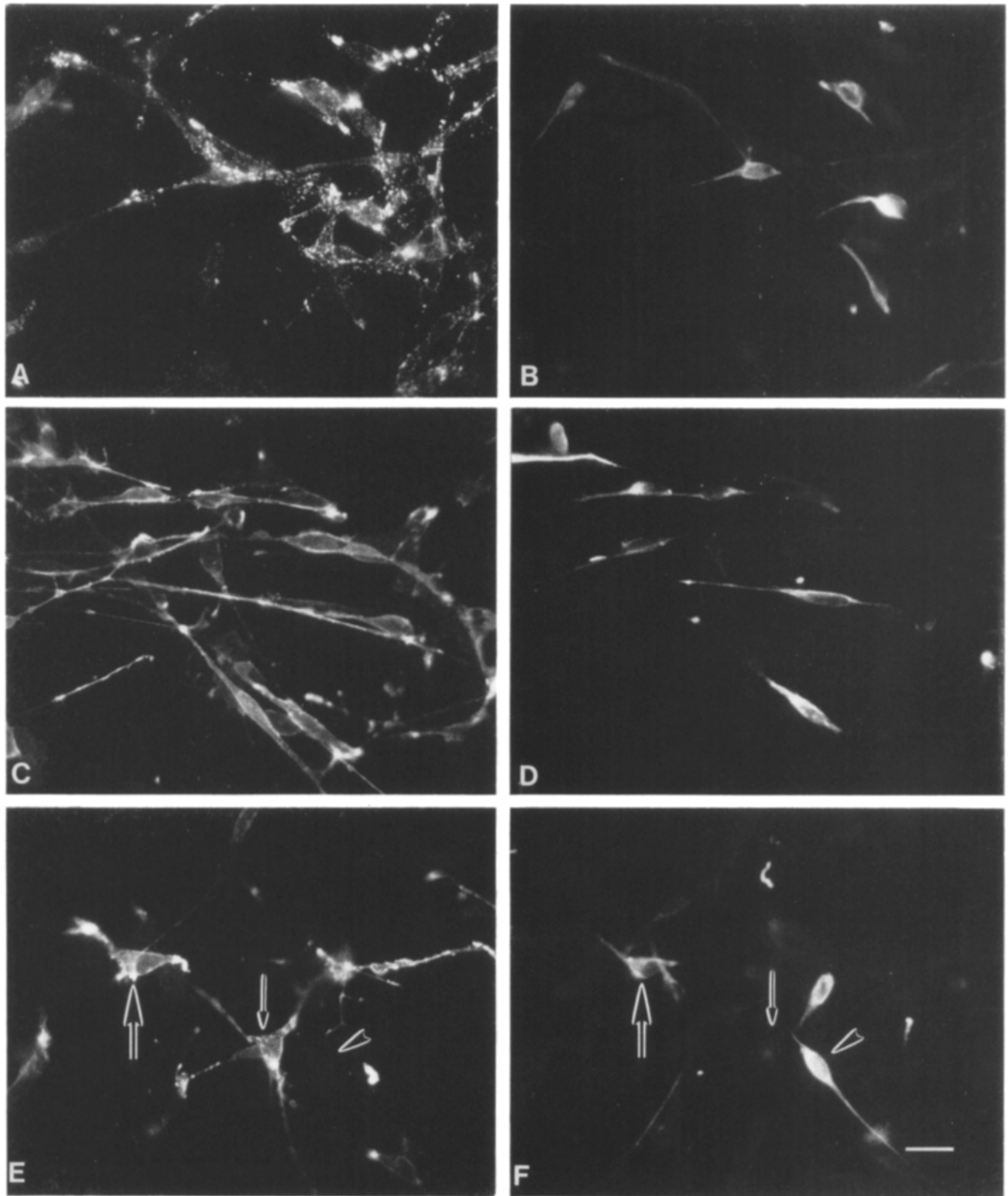


Figure 9. Double-label immunofluorescence localization of NF-M with markers of Schwann cell differentiation. Cells were isolated from 1-d rat sciatic nerve and cultured for 16 h. Visualization of NF-M (*B*, *D*, and *F*) was by rhodamine optics and NGFR (*A*), O4 (*C*), and GalC (*E*) by fluorescein optics. All NF-M⁺ cells were NGFR⁺ and O4⁺. In contrast, three phenotypes were detected in *E* and *F*: GalC⁺,NF-M⁺ (*large arrows*); GalC⁺,NF-M⁻ (*small arrows*); GalC⁻, NF-M⁺ (*arrowheads*). Bar, 20 μ m.

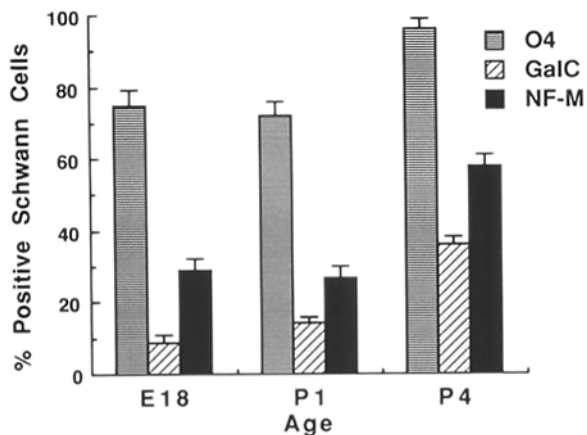


Figure 10. Histogram showing the percentage of Schwann cells expressing O4, GalC, and NF-M at ages E18, P1, and P4. Schwann cells were isolated from sciatic nerves and kept in culture for 16 h before processing for indirect immunofluorescence. The number of Schwann cells as determined by S100 immunoreactivity was: $87 \pm 1\%$ at E18, $86 \pm 2\%$ at P1, and $72 \pm 2\%$ at P4. The increase in the number of GalC⁺ cells from E18 to P4 reflects the differentiation of myelin-forming cells. At P4, 88% of GalC⁺ cells expressed NF-M which shows that NF-M is expressed by myelin-forming cells.

sequence (Napolitano et al., 1987) and each yielded the predicted 1233-bp fragment, as did direct reverse transcription-PCR amplification of total Schwann cell RNA (data not shown). The longest of these clones, pISCP4E3, was fully sequenced and corresponded to the sequence of NF-M (pNF-M2D) published by Napolitano et al. (1987) from bp 550–2717 together with a further 332 bp at the 3' end to the poly A tail. To complete the sequence at the 5' end an overlapping clone pISCP4B8 of 1693 bp was sequenced. The two overlapping clones constituted pISCP4NFM and contained the entire coding region for NF-M with additional sequence at both the 5' and 3' ends. The full-length clone pISCP4NFM was obtained by ligating the Sall/SacI fragment (5' end) of pISCP4B8 to the SacI/NotI fragment (3' end) of pISCP4E3. The resulting construct was subcloned into pSPORT 1 for further analysis. The relationship between the Schwann cell NF-M and that isolated from brain (Napolitano et al., 1987) is shown in Fig. 8.

Developmental Expression of NF-M in Schwann Cells

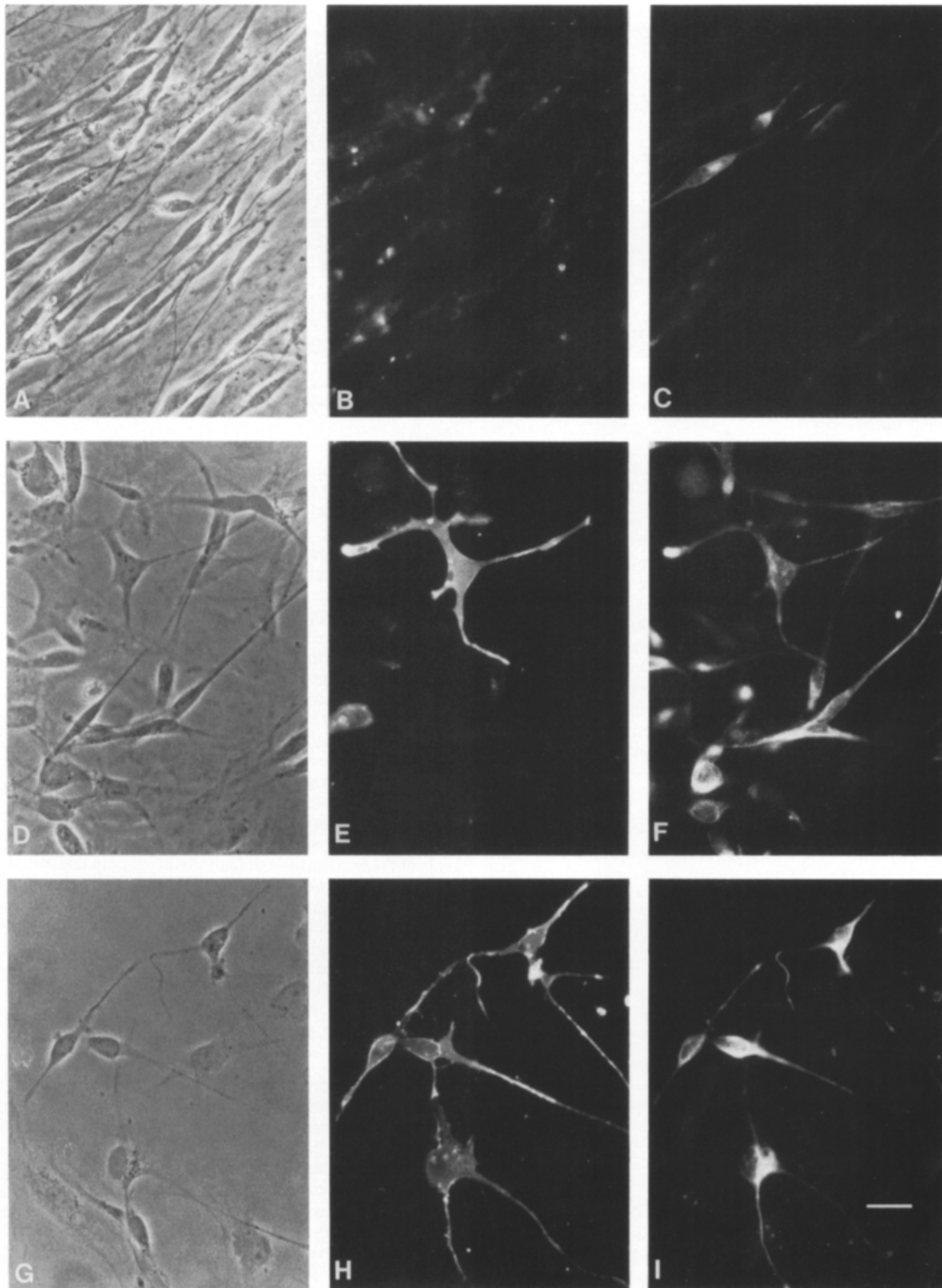
The relationship between NF-M expression and Schwann cell development was investigated by comparing the expression of NF-M with that of the well-characterized differentiation markers: Ran-1 (217c) which is present in most Schwann cells at E18, O4 which appears at E16, and GalC which is first detectable in myelin-forming Schwann cells at E18–19 (Jessen et al., 1990; Mirsky et al., 1990). Since myelination begins in earnest soon after birth (Hahn et al., 1987), we

considered that cells taken from the sciatic nerves of 1-d-old rat pups should contain a significant number of premyelinating and early myelin-forming cells together with a number of non-myelin-forming cells. The cells were cultured for 16 h, and the expression of NGFR, NF-M, O4, and GalC was determined by indirect immunofluorescence. During this period of culture there was no significant dedifferentiation of phenotype as a result of the loss of axonal contact since 31% of the Schwann cells were still strongly P₀ positive.

All of the Schwann cells from 1-d sciatic nerve expressed NGFR whereas much fewer NF-M⁺ cells were present (Fig. 9, A and B). Cells that were NGFR⁺,NF-M⁻ and NGFR⁺,NF-M⁺ were observed but there were no NGFR⁻,NF-M⁺ cells which indicated that NGFR expression precedes that of NF-M. Similarly, O4 expression precedes that of NF-M (Fig. 9, C and D). In contrast, cells stained with the O1 mAb which specifically recognizes GalC displayed three phenotypes: GalC⁻,NF-M⁺, GalC⁺,NF-M⁺, and a relatively small number of GalC⁺,NF-M⁻ (Fig. 9, E and F). These results suggested that NF-M appears during Schwann cell development in the rat sciatic nerve after O4 but somewhat earlier than GalC.

To elucidate the time of NF-M appearance during development, and the relationship between NF-M expression and myelination, Schwann cells were isolated from sciatic nerves at E18, and from nerves at P1 and P4. After 16 h in culture, the cells were double labeled for NF-M and GalC and for NF-M and O4 (Fig. 10). The percentage of cells in these cultures that were Schwann cells was determined by measuring the number of S100⁺ cells in parallel cultures ($87 \pm 1\%$ at E18, $86 \pm 2\%$ at P1, and $72 \pm 2\%$ at P4). At each age, cells had been in culture for 16 h before processing for indirect immunofluorescence. O4 expression, which is first detected in Schwann cells at E16, increased from 69% of Schwann cells on E18 to 96% of Schwann cells on P4 (Fig. 10). This trend was consistent with the increased expression on Schwann cells of O4 with age observed by Jessen and Mirsky (1991). The number of Schwann cells expressing NF-M also showed an increase with age from 29% on E18 to 58% on P4. Our results on GalC expression are difficult to compare with others' work as we used the O1 antibody which is more specific for GalC (Bansal et al., 1990) than is the R-mAb antibody used in other studies (Mirsky et al., 1990). The percentage of Schwann cells expressing GalC increased from 9% on E18, through 14% on P1 to 36% on P4. At P4, three GalC, NF-M phenotypes were observed: GalC⁺,NF-M⁺ ($50 \pm 3\%$), GalC⁺,NF-M⁻ ($7 \pm 2\%$), and GalC⁻,NF-M⁺ ($43 \pm 4\%$). Thus, 88% of the GalC⁺ cells expressed NF-M, which indicates that NF-M is present in myelin-forming Schwann cells. Myelin synthesis begins in earnest on P1 and we found the expected rise in P₀ expression by Schwann cells from 31% on P1 to 56% on P4. Our values for the observed expression of P₀ on P1 and P4 paralleled those for NF-M and support our argument that NF-M is a component of myelin-forming Schwann cells.

Figure 11. Double-label immunofluorescence showing the induction by cAMP analogues of NF-M and GalC in Schwann cells. Schwann cells were isolated from 5-d rat sciatic nerves and cultured for 24 h in Optimem and 10% FCS. They were then cultured for a further 48 h in: (A–C) 10% FCS, (D–F) 10% FCS and 10 μM db-cAMP; (G–I), 10 μM db-cAMP without FCS. The NF-M was visualized by rhodamine optics (C, F, I) and GalC by fluorescein optics (B, E, H). Cells were also visualized by phase contrast optics (A, D, G). Elevated cAMP induces NF-M (F and I) but GalC expression also requires serum deprivation (H). Bar, 20 μm.



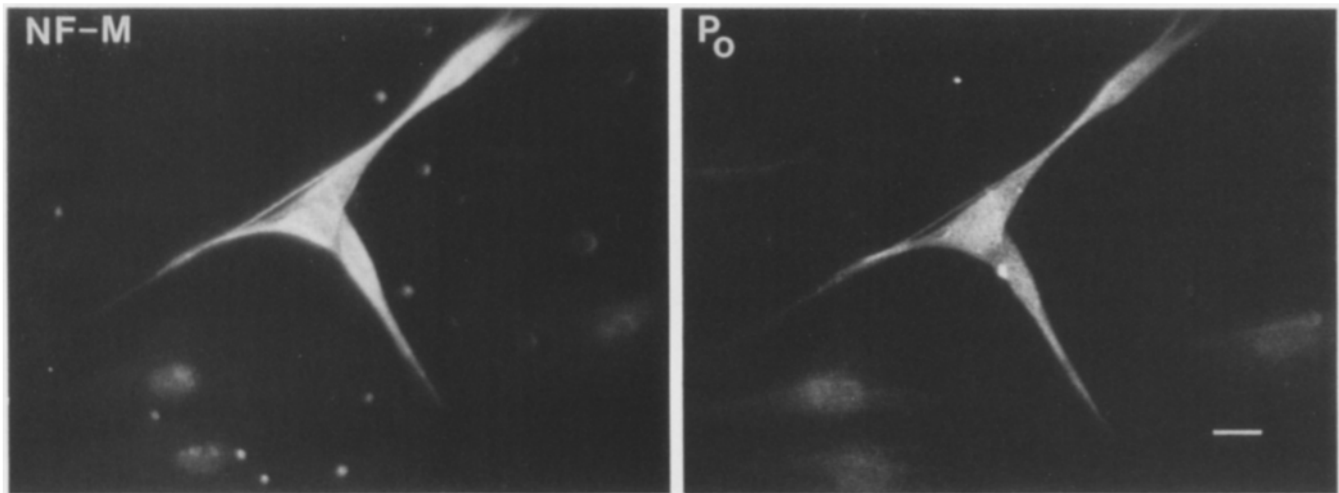


Figure 12. Indirect immunofluorescence of NF-M and P₀ in Schwann cells after treatment with db-cAMP. Schwann cells from 5-d rat sciatic nerve were treated with cAMP analogues for 3 d as in Materials and Methods. Cultures were viewed with rhodamine optics to visualize NF-M and fluorescein optics to visualize P₀. Bar, 10 μm.

Induction of NF-M in Schwann Cells by cAMP Analogues

Schwann cells were isolated from sciatic nerves of rats at P5 and grown in Optimem 1 containing 10% FCS for 24 h before treatments with db-cAMP. When cells were kept for a further 48 h in Optimem containing 10% FCS, they dedifferentiated and very few cells expressed NF-M or were stained with R-mAB which recognizes GalC and the O4 antigen (Fig. 11, B and C). However, if after the initial 24 h of culture cells were kept for 48 h in Optimem containing 10% FCS and db-cAMP, the loss of expression of NF-M was prevented (Fig. 11 F) which suggests that NF-M expression in Schwann cells is under axonal regulation because elevation of intracellular cAMP levels is believed to mimic the effects of axons on Schwann cells (Sobue and Pleasure, 1984a, 1986; Porter et al., 1986).

The change in Schwann cell morphology induced by db-cAMP (Fig. 11 E) is well documented (Sobue et al., 1986; Morgan et al., 1991). If serum were removed after the initial

24 h in Optimem and 10% FCS, and the cells were kept for a further 48 h in Optimem with db-cAMP, expression of NF-M was induced (Fig. 11 I) and all of the NF-M⁺ cells were R-mAb⁺ (Fig. 11 H). P₀ induction can only be induced by cAMP analogues when Schwann cells are quiescent (Morgan et al., 1991), and Fig. 12 shows that these P₀⁺ cells are also NF-M⁺. Thus, NF-M is present in cells expressing myelin proteins. In parallel experiments designed to map the appearance of NF-M expression during Schwann cell development, we found that O4 and NF-M appear in dividing cells and precede the appearance of GalC and P₀ (Figs. 9 and 10) both of which are only expressed in nondividing cells (Morgan et al., 1991). Thus, the results from tissue culture studies were consistent with development in vivo.

Cultured Schwann cells were metabolically labeled in the presence of db-cAMP, and anti-p145 specifically immunoprecipitated a protein of *M_r* 145 (Fig. 13, lane 3). In all three lanes, vimentin and some bands with lower *M_r* were precipitated, even when an irrelevant antibody was used (Fig. 13, lane 1). Antivimentin did not immunoprecipitate NF-M (Fig. 13, lane 2) which is consistent with our finding that this antibody does not recognize NF-M on a Western blot, nor does the anti-p145 recognize vimentin (Fig. 1). The NF-M could not be immunoprecipitated from dedifferentiated cells (data not shown) which supports our contention that NF-M is a protein of differentiating Schwann cells.

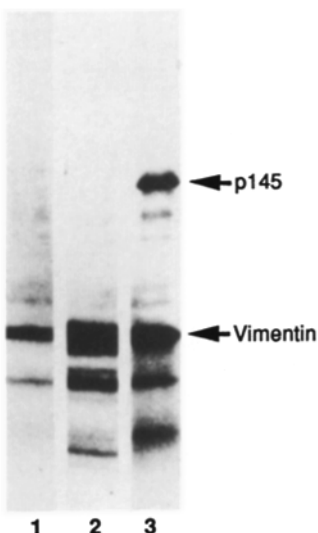


Figure 13. Immunoprecipitation of NF-M from Schwann cells. Schwann cells from 5-d rat sciatic nerve were metabolically labeled for 24 h with [³⁵S]methionine in the presence of 5×10^{-4} M db-cAMP. After extraction of the cells, the cytoskeletons were solubilized in SDS and immunoprecipitates were analyzed by SDS-PAGE. Lane 3 shows a protein with an *M_r* of 145 that was immunoprecipitated with anti-p145 which was not present in immunoprecipitates using nonimmune serum (lane 1) or antivimentin (lane 2).

Discussion

In this paper we have described a component of the IF network of differentiating Schwann cells which we initially called p145 and subsequently identified as NF-M. We have identified p145 as an IF protein because it is exclusively present in the cytoskeleton, it bundles perinuclearly in response to colchicine, and because it can be detected by immunoelectron microscopy in IFs. Axonal contact appears to regulate NF-M expression because it is lost from dissociated Schwann cells in tissue culture and because cAMP, which is believed to mimic some of the effects of axons on Schwann cells, induces re-expression of the protein.

In the rat sciatic nerve, the first Schwann cells appear at E15-16 (Jessen and Mirsky, 1991); in response to axon-Schwann cell contact they begin to express O4 between E16 and E17 and by E20 all Schwann cells express O4 (Jessen et al., 1990). Consistent with these earlier data we found a high number of O4⁺ cells in our cultures from sciatic nerves at E18 (69%), and by P4 essentially all Schwann cells expressed this marker. Myelin-forming cells express GalC at E18-19 whereas non-myelin-forming Schwann cells differentiate later. We found an increase in the percentage of Schwann cells expressing GalC from 9% on E18 to 36% on P4, a result which is consistent with the increase in abundance of cells expressing the myelin protein P₀ during this period (31% on P1 and 56% on P4). The percentage of cells which expressed P₀ on P1 and P4 was greater than that which expressed GalC, indicating that P₀ expression precedes that of surface GalC. The fact that 88% of the GalC⁺ cells also expressed NF-M clearly demonstrated that NF-M is a protein of myelin-forming Schwann cells. Furthermore, we observed that NF-M was retained in Schwann cells that were induced to re-express P₀ (Fig. 12). Further work will be needed in order to delineate the expression of NF-M in non-myelin-forming cells.

The two IF proteins of Schwann cells that have been described so far are vimentin and GFAP. Vimentin is present in both differentiated and undifferentiated Schwann cells (Autilio-Gambetti et al., 1982) and can be detected in Schwann cell cultures from E15 onwards (Jessen et al., 1990). GFAP is first detected on E18 but down-regulates as the myelinating phenotype develops (Jessen et al., 1990). Therefore, we considered the possibility that NF-M replaced GFAP in myelin-forming Schwann cells. We were able to detect GFAP in a small number of Schwann cells at P4 (5%) using the G-A-5 mAb. A much larger number was detectable with a polyclonal anti-GFAP from Dakopatts (38%), which is consistent with the data of Jessen et al. (1990). However, immunoblotting experiments revealed that the Dakopatts antibody was not specific for GFAP, and that it cross-reacted with NF-M, a result that is consistent with the findings of Hansen et al. (1989) who found that this antibody recognizes neurofilaments. When G-A-5 was used, no difference in GFAP immunoreactivity was observed in cultures from 1- and 4-d-old sciatic nerves. Therefore, we suggest that NF-M and vimentin are the main IF proteins of differentiating Schwann cells. There are several precedents for the exchange of IF protein networks during cell differentiation and NF-M may replace GFAP at a very early stage of Schwann cell development in the sciatic nerve (Lazarides, 1982; Cossette and Vincent, 1991; Lendahl et al., 1990; Fliedner et al., 1990; Steinert and Liem, 1990). Myelination necessitates major changes in Schwann cell morphology which must involve the cytoskeleton and the NF-M IF protein may be best suited to the role of sustaining the altered shape which allows axon ensheathment and subsequent myelination.

This paper is the first report of the normal occurrence of an IF protein of the NF type in nonneuronal cells in the nervous system. Although NF proteins are normally restricted to postmitotic neurons, NF-L has been detected in the beta cells of the islets of Langerhans in the embryonic rat, thus raising the possibility that these cells originate in the neural crest (Ecurat et al., 1991). Furthermore, NF proteins have been incorporated into IF-like arrays in nonneuronal cells after transfection (Monteiro and Cleveland, 1990; Chin and

Liem, 1990) and in transgenic mice (Monteiro et al., 1990). It has not escaped our attention that the abundance of NFs in peripheral nerves makes the detection of NF-like proteins in Schwann cells in vivo a difficult task which probably explains why Schwann cell NF-M has been overlooked until now. It should be emphasized however that mRNA encoding NF-M was detected by Northern blotting in sciatic nerve (Fig. 7). Why differentiating Schwann cells should synthesize a NF-like protein remains unclear. Equally puzzling is why Schwann cells synthesize NF-M alone of the NF triplet since these proteins are normally coordinately expressed in neurons. At any rate, the possibility that NF-like proteins exist in other nonneuronal cells should now be considered seriously.

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