Expression of P30, a Protein with Adhesive Properties, in Schwann Cells and Neurons of the Developing and Regenerating Peripheral Nerve

Maryellen M. Daston and Nancy Ratner

Department of Anatomy and Cell Biology, University of Cincinnati Medical School, Cincinnati, Ohio 45267-0521

Abstract. P30 is a heparin-binding protein with adhesive and neurite outgrowth-promoting properties present at high levels in the developing rat central nervous system (Rauvala, H., and R. Pihlaskari. 1987 *J. Biol. Chem.* 262:16625-16635). Partial sequencing of p30 has revealed homology or identity with HMG-1 (Rauvala, H., J. Merenmies, R. Pihlaskari, M. Korkolainen, M.-L. Huhtala, and P. Panula. 1988. *J. Cell Biol.* 107:2292-2305), a 28-kD protein that was originally purified from the thymus (Goodwin, G. H., C. Sanders, and E. W. Johns. 1973. *Eur. J. Biochem.* 38:14-19) which binds DNA in vitro. We have analyzed the distribution of p30 in the developing rat peripheral nervous system (PNS). P30 was detected by immunohistochemistry and Western blot analysis using antibodies raised against intact p30 and against a synthetic peptide corresponding to the amino terminus of the p30 molecule.

P30 was localized to nonnuclear compartments of neurons and peripheral glial cells (Schwann cells). P30 immunoreactivity of PNS neurons persisted into adulthood. In contrast, Schwann cell staining decreased after the second postnatal week and was not detectable in adult animals.

Neuron-Schwann cell contact was correlated with diminished p30 levels in Schwann cells. Schwann cells of the normal adult sciatic nerve did not express p30; however, when deprived of axonal contact by nerve transection, the Schwann cells of the distal nerve stained intensely for p30. In addition, when Schwann cells and dorsal root ganglion neurons were grown in coculture, Schwann cells that were associated with neurites were not as intensely stained by anti-p30 as Schwann cells that were not in contact with neurons.

The pattern of p30 expression during development and regeneration, and its apparent regulation by cell-cell contact suggests that p30 plays a role in the interaction between neurons and Schwann cells during morphogenesis of peripheral nerves.

THE development of the peripheral nervous system (PNS)[1] provides a good example of the importance of cell-cell interactions in morphogenesis. A large body of work has demonstrated the developmental interdependence of neurons and Schwann cells, the major cellular components of the PNS. Schwann cells adhere to neurons (Salzer and Bunge, 1980; Sobue and Pleasure, 1985) and contact with axons stimulates Schwann cells to proliferate (Wood and Bunge, 1975; Salzer et al., 1980; Ratner et al., 1987), to produce basal lamina components (Bunge et al. 1982, Clark and Bunge, 1989), and to form myelin (Weinberg and Spencer, 1975). Schwann cells in turn may affect developing neurons. Schwann cells promote axonal outgrowth (Fallon, 1985; Rickmann and Fawcett, 1985; Tomaselli et al., 1986) and may be involved in guiding axons to their appropriate targets (Yntema, 1943; Noakes and Bennet, 1987; Noakes et al., 1988).

Defined adhesive molecules have been implicated in certain of these neuron-Schwann cell interactions. For example, antibodies that recognize Ng-CAM (L1, NILE) can block ensheathment of axons and myelin formation by Schwann cells in vitro (Seilheimer et al., 1989; Wood et al., 1990). In addition, a combination of antibodies against Ng-CAM, N-cadherin, and integrin, can almost entirely block neurite outgrowth on Schwann cell surfaces (Bixby et al., 1988; Seilheimer and Schachner, 1988). It is not yet known how many adhesive molecules contribute to peripheral nerve morphogenesis. There is evidence that as yet undefined molecules contribute to some aspects of peripheral nerve development. For example, the initial event in the interactions between neurons and Schwann cells is the cell-specific recognition and adhesion between neurons and Schwann cells; this initial cell adhesion is not inhibited by antibodies that have been tested against known adhesion molecules (Bixby et al., 1988; Landmesser et al., 1988).

P30 was recently described as a protein that is abundant in neonatal rat brains and present at lower levels in adult

1. *Abbreviations used in this paper*: CNS, central nervous system; DRG, dorsal root ganglia; PNS, peripheral nervous system.

brain. P30 has been shown to have adhesive and neurite outgrowth promoting activity for embryonic rat brain cells and N18 neuroblastoma cells (Rauvala and Pihlaskari, 1987). Because p30 is present in the central nervous system (CNS), is regulated during development, has adhesive activity, and can promote neurite outgrowth, it was important to determine whether p30 is present in the PNS.

P30 has also been called amphoterin, since the carboxy terminal is highly negatively charged while the amino terminus contains eight of thirteen positively charged residues (Rauvala, 1989). The amino-terminal thirteen amino acids of this 30,000-D protein have been sequenced. This sequence has no homology to known adhesive proteins (Rauvala et al., 1988), but is identical to the NH_2-terminal sequence of HMG-1 (Bustin et al., 1990), a 28,000-D protein expressed in many tissues including brain, suggesting that these proteins are related or identical. In this manuscript we use the name p30, as the work on p30 was the first indication of the potential importance of this protein in the development of the nervous system. HMG-1 was initially described as a nuclear protein associated with chromatin (Goodwin et al., 1973), but it has been localized outside of the nucleus in a number of tissue types (Bustin and Niehart, 1979; Teng and Teng, 1981; Mosevitsky et al., 1989). The function of HMG-1 is unknown.

The availability of the amino-terminal sequence of p30, (Rauvala et al., 1988), enabled us to produce an antipeptide antibody to examine the distribution of p30 in the PNS. In this study we have shown that p30 is expressed in the PNS and that its pattern of expression is altered during development and nerve regeneration. P30 is present in both neurons and Schwann cells during early postnatal development, but is not present in Schwann cells of the adult. After nerve transection, p30 is upregulated in Schwann cells. A tissue culture system (Wood and Bunge, 1975, Ratner et al., 1986) was used to study neuron–Schwann cell interactions in the absence of other cells. With this model we were able to demonstrate that p30 expression in Schwann cells is modulated by contact with neurons. The pattern of expression of p30 during development and regeneration and its regulation by cell–cell contact in vitro suggest that this molecule plays a role in the interactions between neurons and Schwann cells that occur during development.

Materials and Methods

Isolation of P30

P30 was prepared as described in Rauvala and Pihlaskari (1987). Briefly, octyl glucoside–solubilized proteins from P8 rat brain membranes were loaded onto a heparin-Sepharose column (Pharmacia Fine Chemicals, Piscataway, NJ) and eluted using a 0–3 M NaCl gradient. The fractions that contained p30 were determined by ELISA. 50 μl from each fraction were added to individual wells. Anti-p30 peptide (10 μg/ml) was used as the probe to identify p30 containing fractions, which were pooled and purified further by Affi-Gel blue (Bio-Rad, Rockville Ctr., NY) chromatography. P30 accounted for ~90% of the total protein that eluted from Affi-Gel blue at 1.5 M NaCl.

Antibodies

The antibody against S100 was a polyclonal antibovine S100 (Dako Corp., Santa Barbara, CA). The mAb 217c was a gift from Jean De Vellis (UCLA). An mAb recognizing the 160-kD neurofilament polypeptide was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Polyclonal antibodies were generated against a peptide, representing the 14 NH_2-terminal amino acids of p30. The anti-p30 peptide antibodies were prepared as described in Rauvala et al. (1988). Briefly, the peptide was coupled to keyhole limpet hemocyanin (Liu et al., 1979) and injected into rabbits (0.3 mg peptide/rabbit/injection). The antibody was purified from serum by affinity chromatography using the synthetic peptide coupled to EAH-Sepharose (Pharmacia Fine Chemicals) (Rauvala et al., 1988).

Polyclonal antibodies were also generated against intact p30, purified as described above. Affi-gel blue-purified p30 was further purified by nonreducing 12.5% SDS-PAGE. One lane from the gel was stained by Coomassie blue to locate the region of the gel containing p30. The p30-containing regions of the gel were cut out and the gel slabs were homogenized in PBS and mixed with Freund's adjuvant (Difco Laboratories, Detroit, MI) for injection into rabbits (60 μg protein/rabbit/injection).

For immunization against both the peptide and intact p30, primary injections were in complete Freund's adjuvant, and subsequent injections (at 3-wk intervals) were in incomplete Freund's adjuvant.

Immunostaining of Tissue Sections and Cultured Cells

Rat pups at postnatal days 1, 3, 5, 8, 9, 11, 15, 42 and adult rats were killed by an overdose of sodium pentobarbital (Anthony Products Co., Arcadia, CA) and then fixed by perfusion with 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4. Embryos at days 15 and 18 of gestation were dissected to expose the spinal cord then fixed by immersion for 20 h at 4°C in the same fixative that was used for perfusion. After fixation, the spinal cords with attached dorsal root ganglia (DRG) and the sciatic nerves were dissected out and placed in 20% sucrose until the tissue was completely infiltrated (usually overnight). The tissue was then embedded in Tissue-Tek O.C.T. compound (American Scientific Products Div., McGaw Park, IL), frozen in liquid nitrogen-cooled isopentane, and then sectioned at 10 μm on a cryostat (Leitz, 1720 Digital). Tissue was stored at −80°C for up to 3 mo after freezing and before sectioning.

Tissue sections were mounted on glass slides, allowed to dry at room temperature for 10 min, then placed in 2% normal goat serum (Gibco Laboratories, Grand Island, NY) in PBS, pH 7.4. They were then treated with the antibodies; anti-p30 peptide (10 μg/ml), anti-p30 (antiserum 1:4,500), S100 (1:1,000), 217c (1:100) or neurofilament (1:1,000) followed by biotinylated goat antirabbit or goat antimouse (Vector Laboratories, Burlingame, CA). Avidin-biotin peroxidase visualization was then performed using the Vecta-stain Elite kit (Vector Laboratories). In some experiments fluorescently labeled secondary antibodies (goat antirabbit FITC and goat antimouse rhodamine; Kirkegaard-Perry, Gaithersburg, MD) were used to visualize immunoreactivity.

Cultured cells to be stained were grown on 12-mm round glass coverslips (Bellco, Vineland, NJ). The cells were fixed in 4% paraformaldehyde or methanol at −20°C and treated with 10% normal goat serum in Leibovitz's L-15 medium (Gibco Laboratories), then with anti-p30 peptide diluted to 10 μg/ml in the same medium. The coverslips were then treated for peroxidase visualization using the Vecta-stain Elite kit (Vector Laboratories), dried, and inverted on glass slides in DPX mounting medium.

For negative controls, anti-p30 peptide was preabsorbed by diluting the antibody to 10 μg/ml in PBS containing 100 μg/ml synthetic peptide. The antibody was incubated with the peptide at room temperature for 1 h. Normal goat serum was then added and the preabsorbed antibody was applied to tissue sections or cultured cells.

Cell Culture

Neurons were prepared from embryonic day 15 rat DRG as described in Ratner et al. (1985). Schwann cells were prepared from sciatic nerves of perinatal rats by the method of Brockes et al. (1979) with modifications as described in Ratner et al. (1986).

Nerve Transection

Adult rats were anaesthetized by ether. The sciatic nerve was exposed by separating the muscles of the upper hindlimb. The nerve was transected just distal to the sciatic notch and the proximal and distal ends were sewn together, with cut ends abutting, with a suture. After 2 wk the animals were killed by an overdose of sodium pentobarbital (Anthony Products Co.), perfused with 4% paraformaldehyde and prepared for immunohistochemistry.

Preparation of Cell and Tissue Extracts

DRG neurons were grown on 35-mm plastic tissue culture dishes coated

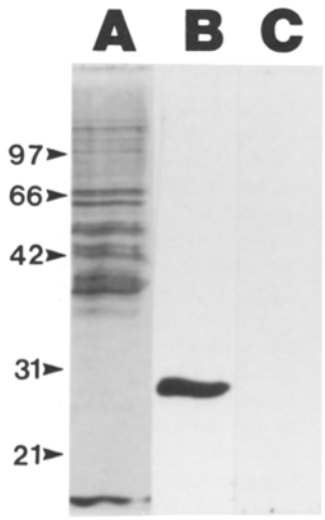


Figure 1. The anti-p30 peptide antibody recognizes a 30-kD brain protein. Octyl-glucoside-solubilized brain membranes from P8 rats were eluted from a heparin-Sepharose affinity column with a NaCl gradient. A p30-containing fraction (determined by ELISA) was separated by reducing SDS-PAGE (10 μg protein/lane) and transferred to nitrocellulose for Western blot analysis. (*A*) The gel was stained with Coomassie blue after transfer to nitrocellulose to show the total protein composition of the heparin-eluted brain extract. The nitrocellulose was incubated with (*B*) 10 μg/ml anti-p30 peptide or (*C*) 10 μg/ml anti-p30 peptide with 100 μg/ ml peptide. Bio-Rad SDS- PAGE low molecular weight standards were used. The anti-p30 peptide antibody is specific for a heparin-binding protein of ~30 kD M_r in young rat brains. Immunoreactivity is abolished by incubation of the antibody with the synthetic peptide that corresponds to the NH_2-terminal amino acid sequence of p30 (Rauvala and Pihlaskari, 1988).

with rat tail collagen (Ratner et al., 1985). The collagen layer containing the cells was scraped up using a plastic pipette tip, then homogenized by 50 strokes in a glass-glass dounce homogenizer in homogenization buffer (HB, 0.16 M sucrose, 1 mM $MgCl_2$ in 10 mM Tris, pH 7.4) with protease inhibitors (100 μg/ml PMSF, 100 μg/ml NEM, and 5 mM EDTA) at 1 ml per ~350 ganglia. The extract was then spun at 2,000 RPM in a clinical centrifuge (Hermle) to remove nuclei and collagen.

Schwann cells grown on tissue culture plastic were suspended in Leibovitz's L-15 medium (Gibco Laboratories) by scraping with a rubber policeman. The cells were pelleted and then extracted in 150 μl/3×10^6 cells PBS with 1% Triton and 0.1% SDS (with protease inhibitors as in HB) for 5 min on ice. The extract was then spun in a microcentrifuge (Allied-Fisher model 253-C) for 2 min to remove nuclei.

Tissue samples from P9 and adult rats, or whole E10 or E12 embryos, were minced with a razor blade, then homogenized in a glass-glass dounce homogenizer in HB with protease inhibitors (1 ml/100 mg tissue). The extract was then centrifuged (as in DRG neuron extract preparation) to remove nuclei.

Protein concentrations of the cell and tissue extracts were determined using the Bio-Rad protein assay.

Western Blots

Extracts were solubilized and proteins separated by 12% SDS-PAGE (Laemmli, 1970; Bio-Rad Protean II slab cell) and transferred to nitrocellulose using the Novablot transfer apparatus (LKB Instruments, Inc., Gaithersburg, MD). The nitrocellulose was then treated with antibodies and processed for alkaline phosphatase visualization (Blake et al., 1984).

Results

Characterization of Antibodies Recognizing P30

An antibody generated against a synthetic peptide corresponding to the NH_2-terminal sequence of p30 (anti-p30 peptide) recognized a 30-kD protein in Western blots of heparin-purified brain extracts, as described by Rauvala et al. (1988). Incubation of the antibody with a 10-fold excess (wt/wt) of peptide abolished reactivity on Western blots, demonstrating that the antibody was specific for the amino-terminal domain of authentic p30 (Fig. 1).

Tissue Distribution of P30

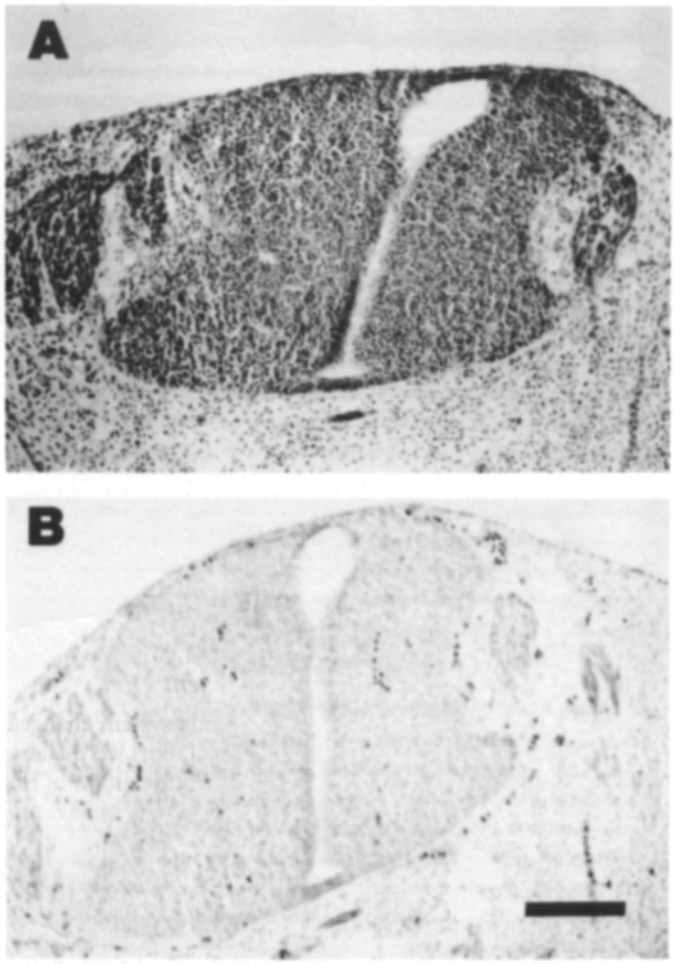
P30 is not specific to the developing nervous system. When entire rat embryos (E10, E12) were sectioned and immunostained using the anti-p30 peptide antibody, all, or almost all, cells of the developing embryo were labeled. Fig. 2 *a* shows an example of an E12 spinal cord, with surrounding mesenchyme and DRG, demonstrating the broad distribution of p30 early in development. Immunostaining was greatly diminished by preabsorption of the antibody with the p30 peptide (Fig. 2 *b*). To confirm that embryonic staining was due to expression of p30, crude extracts of whole E12 embryos were analyzed using Western blots. A band of immunoreactivity was observed that comigrated with authentic brain p30. Immunoreactivity was abolished by incubation of the antibody with the p30 peptide (Fig. 2 *c*). In addition, P9 rat pups contained p30, as analyzed by Western blot, in all organs examined (adrenal gland, kidney, liver, spleen heart, skeletal muscle, sciatic nerve, and brain) (not shown). A wide distribution is consistent with the findings of Rauvala (1989), who found that p30 was present in extracts of P14 rat heart, liver, skeletal muscle, brain, and kidney.

P30 Expression during PNS Development

The distribution of p30 within the nervous system changed during early development. DRG and dorsal roots were examined in detail to determine the developmental pattern of expression of p30 in the PNS. Cryostat sections of spinal cord from the thoracic region, with attached DRG, were taken from rats of various ages ranging from embryonic day 10 (E10) through adulthood. While at E12 p30 immunoreactivity was present in what appeared to be all cells of the developing spinal cord, DRG and surrounding mesenchyme (Fig. 2), postnatally p30 was expressed in only a subset of neurons and glia within the spinal cord (Daston, M. M., and N. Ratner. 1990. *Soc. Neurosci. Abstr.* 16:352). In the developing PNS, postnatal expression of p30 was restricted to neurons and Schwann cells.

Schwann cell proliferation, sorting of axons into fascicles, and myelination of axons by Schwann cells occur during the first two to three postnatal weeks in the rat (Asbury, 1967; Webster et al., 1973); this is the period in which Schwann cell p30 staining is most intense. Immunostaining revealed Schwann cells among the axons of the dorsal root at postnatal day 1 (Fig. 3, *a* and *b*); cell bodies of DRG sensory neurons were also labeled. Schwann cells were consistently labeled by the antibody through postnatal day 11 (Fig. 3, *c* and *d* shows p30 labeling at P3 which is representative of the pattern seen at P3-P11), but no Schwann cell labeling was observed at P15 (Fig. 3, *e* and *f*) or in adult rats (Fig. 3, *g* and *h*). In contrast, DRG neuron cell bodies remained immunoreactive into adulthood. In addition, large axons (or axon fascicles) were stained by the antibody in adult rats. In young animals, axonal staining was sometimes visible proximal to the DRG neuron cell bodies. This pattern suggests that axons are immunoreactive in younger animals, but that low levels of reactivity or the fine caliber of developing axons make these processes difficult to visualize.

Identical staining patterns were observed when the spinal cords and DRG from P8 and adult rats were immunostained using an antiserum against intact p30 purified from P8 rat brain membranes (not shown).



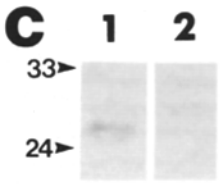


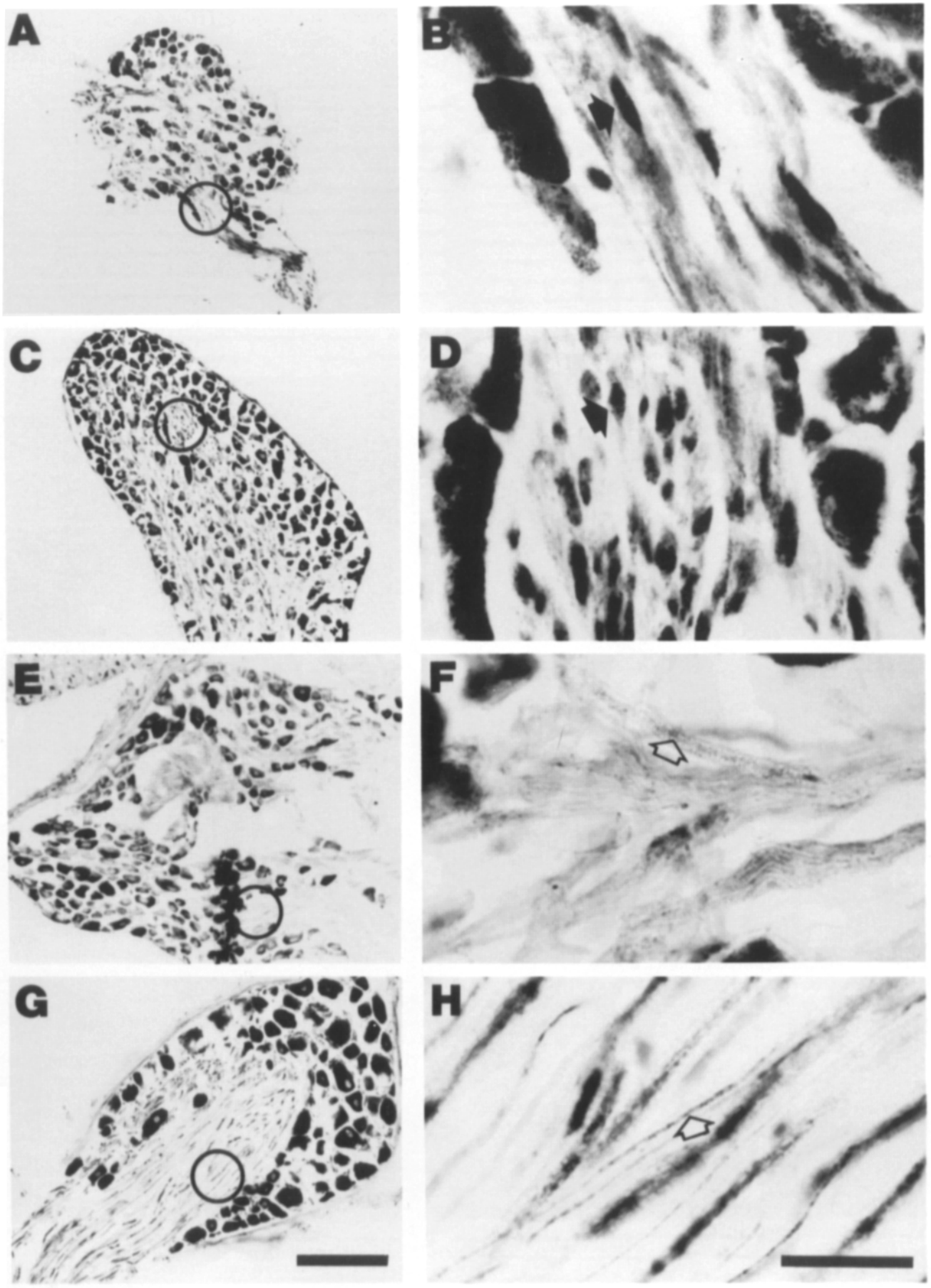
Figure 2. P30 is ubiquitous in embryonic spinal cord and DRG. Adjacent 10-μm cryostat cross sections of an E12 embryo showing the spinal cord, DRG and surrounding mesenchyme were immunostained using (*A*) 10 μg/ml anti-p30 peptide or (*B*) 10 μg/ml anti-p30 peptide with 100 μg/ml peptide. All cells appear to be labeled by the anti-p30 peptide antibody and the intensity of immunostaining is reduced to background levels by preincubation of the antibody with the synthetic peptide corresponding to the NH_2-terminal sequence of p30. (*C*) The presence of p30 in a crude extract of whole E12 embryos (see Materials and Methods) was verified by Western blot (28 μg protein/lane) using 10 μg/ml anti-p30 peptide (lane *1*). P30 immunoreactivity was specifically abolished by incubation of the antibody with the synthetic peptide as in Fig. *1* (lane 2). The band of immunoreactivity comigrated with authentic p30 purified from P9 rat brains (not shown). Bio-Rad prestained SDS-PAGE molecular weight standards were used. Bar, 100 μm.

Expression of P30 in Cultured Neurons and Schwann Cells

Previous studies have shown that p30 is expressed in brain, by dissociated embryonic CNS cells, including neurons, and by a neuroblastoma cell line, (Rauvala and Pihlaskari, 1988). It was therefore surprising to note the apparent expression of p30 by developing nonneuronal cells. However, the interpretation of immunostained cryostat sections of peripheral nerves is complicated by the intimate relationship between axons and their sheath cells. At the light microscope level, therefore, it can be difficult to differentiate between staining of axons or Schwann cells. Therefore, to asses synthesis and expression of p30 in both developing neurons and Schwann cells we turned to an in vitro system in which these cell types can be separated (Wood, 1976; Ratner et al., 1986). The results of two kinds of experiments suggest that p30 is expressed by both DRG neurons and by Schwann cells. First, Western blot analysis of DRG neurons and Schwann cells purified in culture confirmed the presence of p30 in both of these cell types (Fig. 4). Extracts from both neurons and Schwann cells showed a band of immunoreactivity that comigrated with authentic p30 partially purified from p9 rat brains by heparin-Sepharose affinity chromatography. No reactivity was observed when the antipeptide antibody was preabsorbed with 100 μg/ml peptide. Significantly greater levels of anti-p30 immunoreactivity were detected in Schwann cells than in neurons. In addition, immunostaining of cultured DRG neurons and Schwann cells was carried out. Cultured DRG neurons and Schwann cells stained lightly when fixed with paraformaldehyde before immunostaining (Fig. 5, *a* and *c*), or when live cells were incubated with antibody, washed, and then fixed with paraformaldehyde. These results suggest that p30 is expressed by both cell types, and that p30 is detectable on the cell's surface.

Cultures of purified Schwann cells and DRG neurons usually contained a small number of fibroblasts. The fibroblasts were not labeled by the anti-p30 peptide antibody (not shown).

Figure 3. P30 is expressed in Schwann cells of young animals but not in adults. Schwann cells (*solid arrows*) labeled by anti-p30 peptide (10 μg/ml) can be seen in DRG at P1 (*A* and *B*), P3 (*C* and *D*), but not at P15 (*E* and *F*) or in adult (*G* and *H*). *B, D, F,* and *H* are enlargements of the encircled areas in *A, C, E* and *G*, respectively. Open arrows point to large myelinated axons. Bars: (*A, C, E,* and *G*) 50 μm; (*B, D, F,* and *H*) 10 μm.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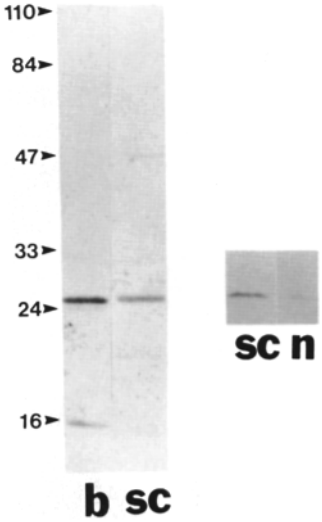

Figure 4. P30 immunoreactivity can be detected in extracts of DRG neurons and Schwann cells purified in culture. Crude extracts of DRG neurons and Schwann cells (see Materials and Methods) were separated by SDS-PAGE and transferred to nitrocellulose for Western blot analysis. A protein with the same mobility as authentic p30 from P9 rat brain (*b*) was labeled by anti-p30 peptide in Schwann cell (*sc*) and DRG neuron (*n*) extracts (50 μg protein/lane). Bio-Rad prestained SDS-PAGE molecular weight standards were used.

Intracellular Expression of P30

Several lines of evidence suggest a significant intracellular pool of p30. The appearance of labeled cells in immunostained tissue sections was indicative of an intracellular antigen. Label was uniform and not confined to the perimeter of the cells (Figs. 2, 3, and 6). When immunostaining cultured cells, permeabilization using several different reagents (methanol, 0.2% Triton-X 100, or 0.1% saponin) before the addition of the anti-p30 peptide antibody always resulted in more intense labeling of Schwann cells and neuron cell bodies as compared to immunostained cells that were not permeabilized. However, when DRG neurons and Schwann cells purified in culture were separated into crude membrane and cytosolic fractions, p30 was detectable by immunoblot in the membrane fractions and not the cytosolic fractions of both cell types (data not shown).

Rauvala et al. (1988) also found evidence for intracellular

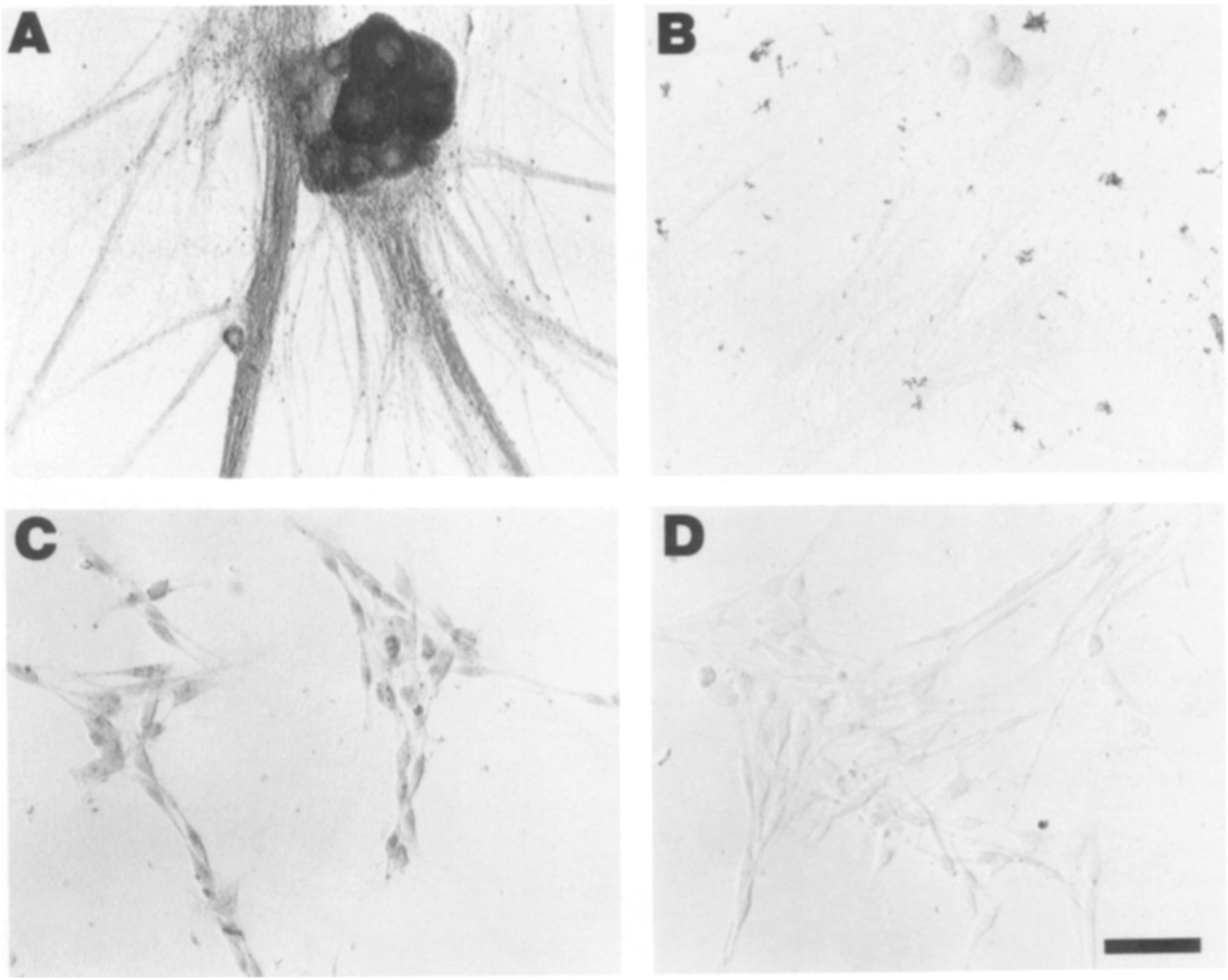

Figure 5. Antibodies against p30 label cell surface of neurons and Schwann cells in culture. DRG neurons and Schwann cells were purified in culture separately. DRG neurons (*A* and *B*) and Schwann cells (*C* and *D*) were fixed in 4% paraformaldehyde (10 min, room temperature) and immunostained using anti-p30 peptide. (*A* and *C*)), or anti-p30 peptide preabsorbed with the peptide (*B* and *D*). Bar, 20 μm.

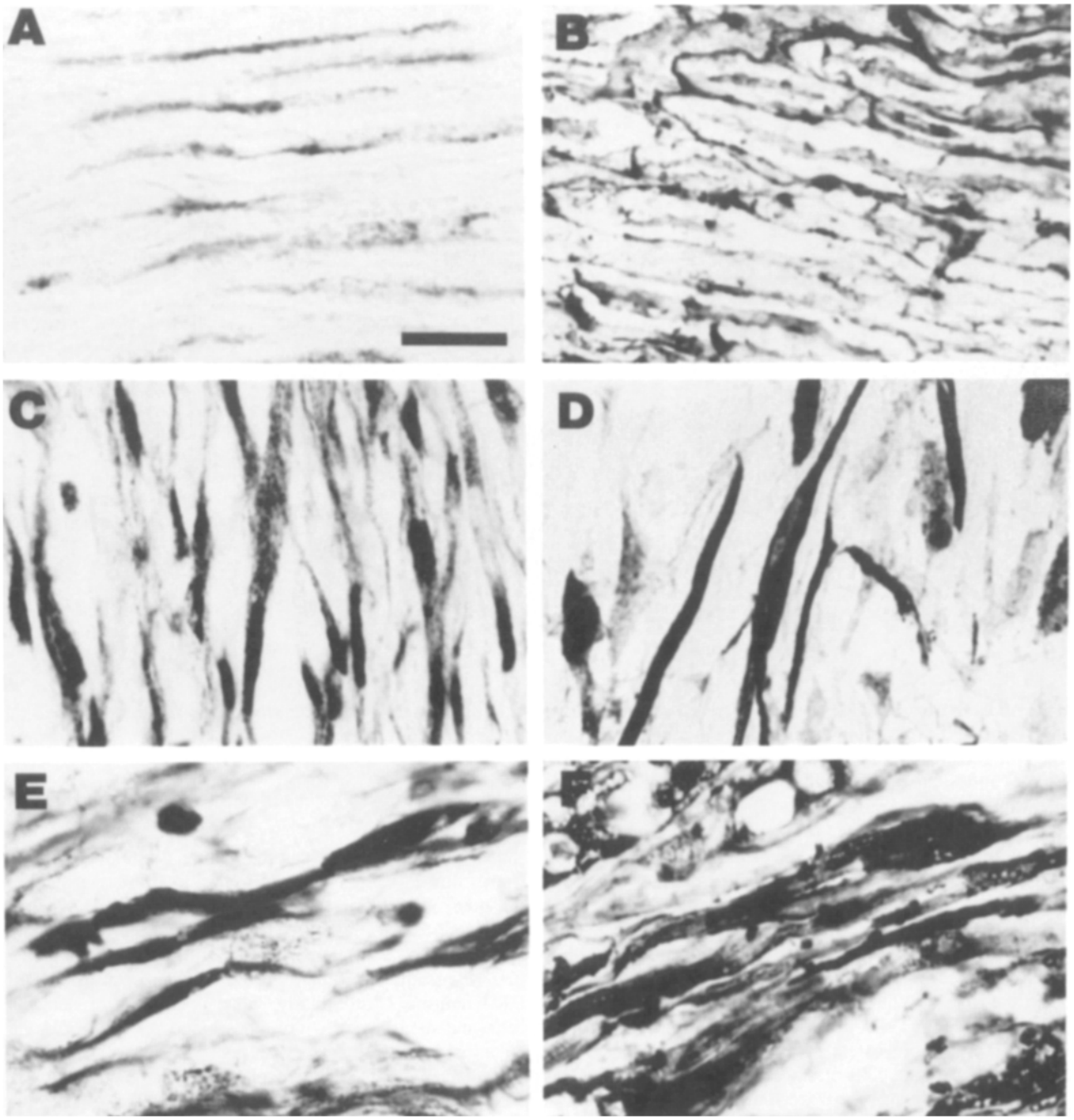

Figure 6. P30 staining increased in Schwann cells of the distal stump of the transected sciatic nerve. 10-μm cryostat sections of proximal stump (*A* and *B*), nerve scar (*C* and *D*), and distal stump (*E* and *F*) of the transected sciatic nerve were immunostained using anti-p30 peptide (*A*, *C*, and *E*)), or anti-S100 (*B*, *D*, and *F*). S100 is a Schwann cell marker. The distribution of p30 and S100 is different in the proximal stump and similar in the nerve scar and the distal stump, indicating that p30 is present in Schwann cells distal to, but not proximal to, the point of transection. Bar, 5 μm.

and cell surface p30. They demonstrated by immunoelectron microscopy of cultured embryonic rat brain cells that p30 was associated with the plasma membrane and intracellularly with polyribosomes.

Modulation of P30 Levels in the Transected Sciatic Nerve

Because nerve regeneration in many ways recapitulates the process of development (Fawcett and Keynes, 1990), it was of interest to examine p30 expression in the injured sciatic nerve, a well-characterized model of peripheral nerve regeneration. The pattern of p30 immunoreactivity of the adult sciatic nerve paralleled that of the dorsal root; axons were lightly stained and no staining of Schwann cells was apparent. However, 2 wk after unilateral nerve transection in adult rats, the pattern of immunostaining was dramatically changed. Proximal to the cut, the pattern of staining for p30

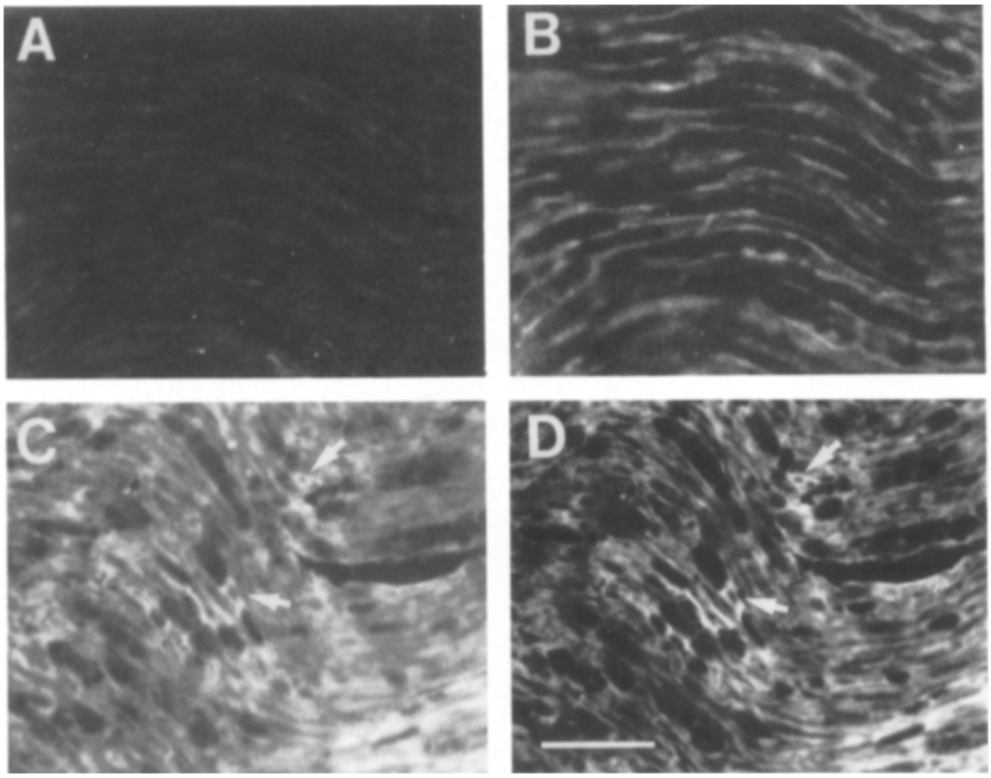

Figure 7. Double labeling of normal and transected sciatic nerve shows that p30 immunoreactivity in the transected, but not in normal nerve, colocalizes with a Schwann cell marker, 217c. 10-μm cryostat sections of normal (*a* and *b*) and the distal stump of transected nerve (*c* and *d*) were double labeled with anti-p30 peptide and 217c. The sections were viewed with fluorescein optics and rhodamine optics to visualize p30 (*a* and *c*) and 217c (*b* and *d*) immunoreactivity, respectively. Arrows point to some examples of cells labeled by both anti-p30 peptide and 217c. Bar, 20 μm.

was identical to the normal nerve (Fig. 6 *a*). However, in the distal stump p30 expression was markedly increased. Distal to the cut, cells that expressed p30 could be seen in the scar (Fig. 6 *c*) and in the nerve distal to the scar (Fig. 6 *e*). P30-containing cells within the scar had a disordered appearance. In contrast, distal to the scar p30 immunoreactivity was in a parallel linear array characteristic of the tubes of extracellular matrix and Schwann cells (bands of Bungner) formed in the regenerating nerve prior to regrowth of axons (Dyck et al., 1984). Fig. 6 *b*, *d*, and *f* show sections, adjacent to those in Fig. 6, *a*, *d*, and *e*, respectively, immunostained with an antibody against S100 that specifically labels Schwann cells in the PNS (Steffansson et al., 1982). Proximal to the point of transection, anti-p30 peptide and anti-S100 label different elements, consistent with the observation that normal adult rat Schwann cells do not contain significant levels of p30. Distal to the cut, the pattern of staining was similar for S100 and p30, suggesting that Schwann cells are the cellular elements responsible for the increase in p30 immunoreactivity seen in the distal stump of the regenerating sciatic nerve. To confirm this, sections of normal sciatic nerve and the distal stump of the transected nerve from the same animal were double labeled with anti-p30 peptide and 217c, an mAb specific for Schwann cells in the regenerating nerve (Fields and Dammerman, 1985) which has been shown to recognize nerve growth factor receptor (Fiori, M. S., G. Ferrari, M. Fabris, S. D. Skaper, P. Polato, and Q. Yan. 1990. *Soc. Neurosci. Abstr.* 16:826). The patterns of immunoreactivity for anti-p30 peptide and 217c were very different in the normal nerve (Fig. 7, *a* and *b*), but they were identical in the transected nerve (Fig. 7, *c* and *d*). Neurofilament immunostaining of the severed sciatic nerve showed that axons had not yet reentered the distal stump; the front of growing axons could be seen at the proximal edge of the nerve scar (not shown).

Modulation of P30 Levels in Schwann Cells by Contact with Neurons

Since Schwann cells deprived of axonal contact by nerve transection increased p30 expression in situ, we wondered if similar modulation would be observed in vitro. Schwann cells were therefore cultured alone or together with purified DRG neurons. Schwann cells stained much more darkly for p30 when cultured separately than when cocultured with DRG neurons. Additionally, when cocultures of Schwann cells and neurons were immunostained for p30, Schwann cells that were associated with neurites were stained much less intensely than Schwann cells on the same coverslip that were not in contact with neurons (Fig. 8). The time course of this change in p30 expression by Schwann cells was analyzed by immunostaining of cocultures in which Schwann cells were added to neurons 3 h, 24 h, 2, 5, 10, or 14 d before fixation. Downregulation of p30 in Schwann cells was observed as early as 3 h after the Schwann cells were added to neurons, and persisted for the duration of the experiment (2 wk). This effect was observed when cells were fixed with methanol before immunostaining, but not when paraformaldehyde was used as the fixative, suggesting that the differences in staining intensity were due to changes in intracellular p30 concentration or distribution. The rapid change in p30 expression following cell–cell contact suggests that p30 responds to or mediates some aspect of neuron–Schwann cell communication. This change in p30 expression is significantly faster than the downregulation of p30 expression by Schwann cells that occurs over several weeks in developing

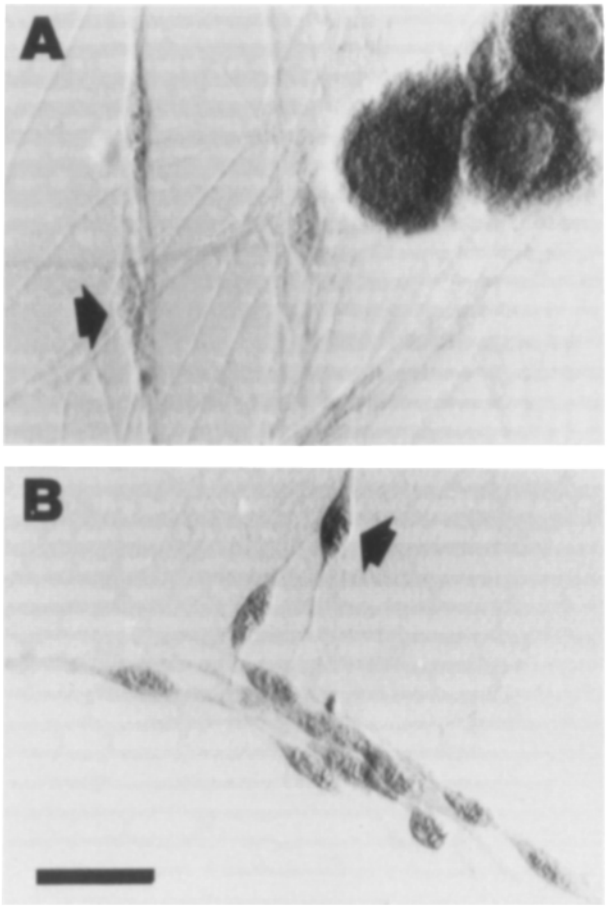

Figure 8. Schwann cells in coculture with DRG neurons stain more intensely for p30 when they are not in contact with neurons. Schwann cells and DRG neurons were purified separately (see Materials and Methods). Schwann cells were added to the neuron cultures and, in this example, incubated together for 24 h. The cells were fixed in methanol (5 min, −20°C) and immunostained using 10 μg/ml anti-p30 peptide. Schwann cells associated with neurites (*A*) were not stained as intensely as Schwann cells from the same coverslip that were not in contact with neurites (*B*). Bar, 10 μm.

nerves. This discrepancy between in vivo and in vitro phenomena raises the possibility that components present in nerves can retard down regulation of p30.

Discussion

The localization of p30 to both PNS neurons and Schwann cells, and its dynamic expression during development demonstrate that p30 is present at the appropriate times and places to participate in the interactions between neurons and Schwann cells which lead to the formation of peripheral nerves. Sensory neurons express p30 throughout development and in adult animals. In contrast, p30 expression in Schwann cells is limited to immature animals and corresponds to an active period of PNS morphogenesis in which Schwann cells proliferate, modify their spatial relationships with growing neurites, and differentiate into ensheathing or myelinating glia (Asbury, 1967; Webster et al., 1973). P30 expression by Schwann cells is diminished in older animals where Schwann cells are differentiated and axon–Schwann cell relationships are established and stable.

Additional evidence suggesting that p30 plays a role in establishing neuron–Schwann cell interactions is the observed change in p30 expression after peripheral nerve transection. The PNS shows a unique capacity for regeneration. After damage to a peripheral nerve, neuronal processes degenerate, leaving Schwann cells without neuronal contact. After injury, Schwann cells modify the expression of a number of molecules in a manner consistent with their regulation during development. For example, N-CAM and Ng-CAM are expressed in all Schwann cells of the embryonic PNS but only in nonmyelinating Schwann cells at later developmental times (Faissner et al., 1984; Nieke and Schachner, 1985). N-CAM and Ng-CAM increase in both axons and Schwann cells in peripheral nerves following injury and return to normal levels after reinnervation (Daniloff et al., 1986; Jessen et al., 1987). Other antigens, such as the myelin-specific molecules P0, MAG, P1, and P2 that are, conversely, expressed at low levels during development and upregulated in mature animals, are downregulated after nerve injury and are not reexpressed until regeneration is complete (Willison et al., 1988). There is evidence that reestablishment of normal nerve morphology depends on interactions with Schwann cells similar to the cell–cell interactions that occur during normal PNS development and that the Schwann cell response to injury is important in providing a permissive environment for axonal regrowth (Kromer and Cornbrooks, 1985; Smith and Stevenson, 1988; Dusart et al., 1989). Therefore, upregulation of p30 in Schwann cells of adult rats after nerve injury is consistent with a role for p30 in PNS morphogenesis and in PNS repair.

What is the function of p30 in peripheral nerve development? We have shown that p30 is expressed by most or all cells in early embryos, and therefore is expressed by cells originating from more than one germ layer. The wide distribution of p30 during development suggests the possibility that p30 functions in cellular processes common to all developing organs. Previous work on the heparin-binding molecule, p30, suggested that this abundant protein might have an adhesive function, since embryonic CNS neurons bind to immobilized p30 in a dose-dependent, saturable manner, and binding can be blocked by specific antibodies that recognize p30. In addition, immobilized p30 promotes neurite outgrowth from this same population of neurons (Rauvala and Pihlaskari, 1987). These results and our finding that p30 expression is increased during PNS pattern formation are consistent with the hypothesis that p30 is involved in adhesion, but do not rule out other possible functions.

While adhesive molecules are expected to be found on cell surfaces and/or in the extracellular matrix, P30 is detectable at low levels on the cell surface and at higher levels inside cells. Lactoperoxidase catalyzed iodination of live brain cells labeled p30 (Rauvala et al., 1988), providing evidence that p30 is associated with cell surfaces. In addition, Rauvala and Pihlaskari (1987) localized p30 to both the cytoplasm and the cell surface of cultured rat embryonic brain cells using immunoelectron microscopy. Similarly, while we observed low levels of p30 immunoreactivity on paraformaldehyde-fixed and live-stained DRG neurons and Schwann cells, immunoreactivity was significantly stronger after perme-

abilization of neurons or Schwann cells using either methanol or detergent.

The finding that brain cells bind to (and extend neurites on) immobilized antibodies recognizing p30 corroborates that p30 is present on the surface of cells and is available for binding to ligands (Rauvala et al., 1988). If p30 functions as an adhesive molecule, the subcellular localization of p30 predominantly to the cytoplasm was unexpected. However, it has been proposed that secreted adhesive molecules might mediate adhesive interactions by binding ligands on adjacent cells or on a cell and the extracellular matrix (Regen et al., 1986; Rutishauser and Jessel, 1988). Such an activity is feasible for p30. P30 inside Schwann cells is downregulated by neuronal contact in cell culture, consistent with the possibility that in response to neuronal contact p30 is secreted from Schwann cells and binds to cell surface receptors on neurons and/or glial cells, and in this way mediates neuron–Schwann cell adhesion. While we cannot yet rule out that changes in p30 immunoreactivity are due to obscuring of immunoreactive epitopes or changes in transcription or translation of p30, the time course of this change is extremely rapid, and thus is consistent with p30 secretion by Schwann cells. The initial adhesive interaction between neurons and Schwann cells has not been accounted for by the activity of any known CAM (Ratner et al., 1986; Bixby et al., 1988; Seilheimer and Schachner, 1988), which leads to the speculation that p30 may be involved.

Intracellular p30 released by cell–cell contact and/or other cues would become available to bind cells that bear specific receptors on their cell surface. Rauvala has proposed that p30 is a peripheral membrane protein associated with the cell surface via heparin-like molecules (Rauvala et al., 1988), since addition of heparin to cultured brain neurons displaced p30 from the cell surface. The extremely high positive charge ratio in the amino terminus of p30 is consistent with interaction with heparin; the p30 amino terminus contains a heparin-binding consensus sequence as defined by Cardin and Weintraub (1988). Cell surface heparan sulfate proteoglycans are present on both neurons (Ratner et al., 1985; Needham et al., 1988) and Schwann cells (Carey and Evans, 1989), and are therefore candidate receptors for p30.

The amino-terminal sequence of p30 is identical to that of the broadly distributed DNA-binding protein HMG-1 (Tsuda et al., 1988; Wen et al., 1989). Other lines of evidence suggest that p30 and HMG-1 are similar or identical. HMG-1 has been shown to have a molecular mass of 28 kD by electrophoresis, sedimentation coefficient, and amino acid sequencing (Walker et al., 1980); this mass has been confirmed by molecular cloning (Tsuda et al., 1988; Wen et al., 1989). P30 also migrates with an apparent mobility of ~28 kD (see Fig. 1, 2, and 4). In addition, Rauvala (1989) has found that, like HMG-1 (Cary et al., 1983), the COOH-terminal of p30 contains a cluster of negatively charged amino acids. Finally, while HMG-1 was initially purified from calf thymus nuclei (Goodwin et al., 1973) in a variety of tissues it has been localized predominantly or exclusively to nonnuclear subcellular fractions, giving rise to the speculation that it is not merely being stored for use in the nucleus, but that HMG-1 may function in cellular processes outside of the nucleus (Bustin and Neihart, 1979; Teng and Teng, 1981; Mosevitsky et al., 1989). While HMG-1 has not previously been studied in the PNS, our results suggesting nonnuclear localization of p30 are consistent with results obtained for HMG-1 in the central nervous system. Subcellular fractionation of adult brain showed that HMG-1 was not detectable in nuclei (Mosevitsky et al., 1989). Clarification of the relationship between p30 and HMG-1 will not be possible until the complete sequence of p30 is known. It is important to note that both the peptide antibody and the antibody against p30 recognize a single band on Western blots and do not recognize any material in nuclear fractions (not shown) consistent with both the identity of these molecules and the nonnuclear localization of HMG-1 in nervous tissue seen in previous studies (Mosevitsky et al., 1989).

While the relationship between p30 and HMG-1 does not preclude an adhesion-related function, it does suggest additional or alternative functions. Several functions for nuclear HMG-1 have been proposed based on its association with DNA. While we have not observed any p30 immunoreactivity in nuclei in these studies, it is possible that p30 is in the nucleus at concentrations below the limit of detection by immunohistochemistry or that it is modified in the nucleus in a way that masks epitopes recognized by our antibodies. HMG-1 may facilitate transcription in a tissue-specific manner (Eink and Bustin, 1983; Kleinschmidt et al., 1983). If p30 has the same properties it could affect expression of genes that are important in the neuronal contact-induced differentiation of Schwann cells. HMG-1 levels and cytoplasmic/nuclear ratios have been shown to change with both state of proliferation and differentiation in oviduct and liver (Teng and Teng, 1981; Mosevitsky et al., 1989). Our results suggest that cellular expression of p30 is regulated during changes in the state of differentiation of Schwann cells. The regulation of Schwann cell phenotype by contact with axons suggests that a novel mechanism of HMG-1-like protein regulation might be through cell–cell contact.

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Note Added in Proof. We have recently found that an antibody against HMG-1/2 obtained from Dr. Michael Bustin (National Cancer Institute, NIH) (*a*) reacts with p30 in Western blot analysis; (*b*) like anti-p30, stains Schwann cells in culture on and off neurites differentially; and (*c*) shows no nuclear staining of Schwann cells or neurons.

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