Neurons Regulate Schwann Cell Genes by Diffusible Molecules

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Abstract. Successful peripheral nerve regeneration and functional recovery require the reestablishment of the neuron–Schwann cell relationship. In the regenerating rat sciatic nerve, neurons differentially regulate Schwann cell genes. The message for the low-affinity NGF receptor, p75<sub>NTR</sub>, is induced in Schwann cells distal to the injury and is repressed as regenerating axons make contact with these cells. The inverse is true for mRNA of the myelin gene P<sub>0</sub>; expression decreases distal to injury and increases as new axons contact Schwann cells and a program of myelination is initiated. Using an in vitro co-culture paradigm in which primary neurons and adult Schwann cells are separated by a microporous membrane, we show that axon contact is not an absolute requirement for neuronal regulation of Schwann cell genes. In this system neurons but not other cell types, repress the expression of Schwann cell p75<sub>NTR</sub> while inducing the expression of the POU domain transcription factor, suppressed cAMP inducible POU, and myelin P<sub>0</sub>. These results demonstrate that regenerating axons can direct the Schwann cell genetic program from a distance through diffusible molecules.

The peripheral nerve Schwann cell supports and myelinates the neuron it subserves. When the established neuron–Schwann cell relationship is disrupted by a crush injury of the sciatic nerve, a series of well-defined cellular and molecular changes occur in the nerve (8) which result in the re-establishment of that relationship. Schwann cells, that have responded to injury by proliferation (19, 40), absorption of myelin sheath debris (14), and synthesis of neurotrophins (13), must remyelinate regenerated axons before functional recovery is achieved. Defining mechanisms through which the neuron–Schwann cell relationship is reestablished during regeneration may permit intervention to increase functional recovery of injured nerve.

Several Schwann cell genes are known to be differentially regulated during regeneration. mRNA encoding NGF (13, 27), p75<sub>NTR</sub> (13), Gla Maturation Factor β (4), and neural cell adhesion molecule (N-CAM) (15) increase distal to a crush injury. The expression of the myelin genes; myelin-associated glycoprotein (10, 30), myelin basic protein (9, 20, 46), myelin P<sub>0</sub> (9, 24), and PMP22 (7, 44) decreases post-crush. Successful regeneration is accompanied by remyelination and a return to normal expression levels. Regenerating neurons are thought to regulate the expression of Schwann cell p75<sub>NTR</sub> (18, 45) and myelin P<sub>0</sub> (20, 24, 37). This gene regulation, as well as Schwann cell proliferation, apparently requires direct contact between neurons and Schwann cells (20, 41). For example, no Schwann cell proliferation occurs if these cells are separated from neurons by a permeable membrane (42). We have reexamined the requirement of neuron–Schwann cell contact for gene regulation in an in vitro paradigm in which confluent Schwann cells isolated from the injured adult sciatic nerve are co-cultured with primary neurons in the absence of neuron–Schwann cell contact. This system mimics regeneration in adult animals and permits the investigation of neuronal regulatory effects. In this report we show that neuronal co-culture results in a diminution in expression of p75<sub>NTR</sub> and an induction of suppressed cAMP inducible POU (SCIP) (32) and myelin P<sub>0</sub> expression in Schwann cells through a diffusible interaction. This regulatory function is neural specific with other embryonic cells having no effect on these Schwann cell genes. This data indicates that neurons can influence Schwann cell genetic programs from a distance. Further, the data proves that axon contact is not an absolute requirement for neuronal regulation of Schwann cell p75<sub>NTR</sub> and myelin P<sub>0</sub> expression.

Materials and Methods

Schwann Cell Culture

Activated adult Schwann cells were isolated as described (3). Briefly, adult male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of ketamine (5 mg/kg) and 30% chloral hydrate (15 mg/kg). Bilateral 5 mm, 45° incisions through muscles of the upper hindlimb were made and sciatic nerves were exposed at the sciatic notch. Nerves were crushed by compression with fine jewelers' forceps for three 15-s pulses. Wounds were clamped and the animals recovered. After 30 h, the injured rats were eu-
thanized in a CO₂ atmosphere. Using sterile procedure, 5 mm of the sciatic nerve distal to the crush was quickly removed to calcium-, magnesium-free (CMF) PBS. The epineural sheaths were stripped and the nerves were minced to 0.5-1 mm size. The CMF-PBS was removed and 0.15% collagenase A in CMF-PBS was added. The digest was incubated at 37°C in 5% CO₂ atmosphere. After 1 h, trypsin was added for a final concentration of 0.015%. After an additional 30-min incubation, the digest was triturated five times through a 19-gauge syringe. The digest was diluted with 20 ml culture medium (DME + 10% heat-inactivated horse serum + 100 U/ml penicillin + 100 μg/ml streptomycin). This suspension was pelleted at 1,200 rpm/3 min, the digestion solution was removed, and the cells were resuspended in 10 ml culture medium. This cell suspension was passed through 60-μm pore mesh and plated. Cells were allowed to adhere for 1 h at 37°C, and then unattached cells were replated as before. After a third passage, non-adherent cells were passed to 35 mm plates that had been precoated with poly-L-ornithine (1 mg/ml) and incubated at 37°C, and then unattached cells were replated as before. After a third passage, non-adherent cells were pelleted and counted. The remaining adherent cells (fibroblasts) were cultured over-night, removed from plates and counted.

**Primary Embryonic Chick Cells**

E8 (Hamburger–Hamilton stage 34–35) White Leghorn chick embryos (11) were sacrificed by decapitation under sterile conditions. **Dorsal Root Ganglia Culture.** The body cavity was eviscerated and the spinal cord was exposed. Fine forceps were used to pluck 20–30 dorsal root ganglia (DRG) from the vertebral column of each embryo and place them in CMF-PBS. The ganglia were pelleted at 600 rpm for 3 min. PBS was removed, ganglia were resuspended in 0.08% trypsin, and then incubated at 37°C circulating water bath for 10 min. A 2× volume of medium (DME + 10% heat-inactivated horse serum + penicillin/Streptomycin) was added, the DRG were triturated 5× with a flame-polished glass pipette and trituration was repeated with a smaller bore flame-polished pipette. Cells were pelleted as before, resuspended in medium, passed through nylon and preplated for 2 h on plastic at 37°C with 5% CO₂. Contaminating non-neuronal cells accounted for <1% of the total cell population. The non-adherent neurons were pelleted as before, resuspended in serum-free DME, and counted. The remaining adherent cells (fibroblasts) were cultured overnight, removed from plates and counted.

**Retina.** Chick E8 eyes were removed and sheets of neural retina cells were separated from the pigmented epithelium and placed in CMF-PBS. After mechanical disruption by trituration with a flame-polished glass pipette, trituration was repeated with a smaller bore flame-polished pipette. Cells were pelleted as before, resuspended in medium, passed through nylon and preplated for 2 h on plastic at 37°C with 5% CO₂. Contaminating non-neuronal cells accounted for <1% of the total cell population. The non-adherent neurons were pelleted as before, resuspended in serum-free DME, and counted. The remaining adherent cells (fibroblasts) were cultured overnight, removed from plates and counted.

**Liver.** Chick E8 liver was removed to CMF-PBS, mechanically disrupted by trituration and cells isolated as described above for retina.

**Co-culture.** Confluent Schwann cell cultures were washed twice with serum-free DME and 30-mm micellilis inserts (Millipore Continental Water Systems, Bedford, MA) that had been precoated with laminin (10 μg/ml) were placed within the wells in a final volume of 3 ml. Cell suspensions at a concentration of 10⁶ cells/500 μl were added to each insert. The cocultures were incubated for 2–6 d after which Schwann cell total cellular RNA was isolated.

**Scanning Electron Microscopy**

Schwann cell–E8 DRG cocultures were grown as described. After 2 d, milllicells were fixed in 2% glutaraldehyde and prepared for scanning EM by the method described (36). Microporous membranes were removed from their plastic support, the top or cellular side of the milllicells were observed first and then turned to observe the underside. Specimens were examined with a Philips 505 scanning electron microscope.

**Northern Blot Analysis**

Total cellular RNA was purified from normal rat sciatic nerve as described (6). Primary Schwann cell RNA was prepared using the guanidinium thiocyanate procedure (6). RNA was resuspended in diethylpyrocarbonate (DEPC)-treated water and quantitated by OD₂₆₀/₂₅₀. Methods used for electrophoresis of RNA, transfer to nylon (Hybond N [Amersham, IL]) and Northern blot hybridization were as described (28). The amounts of RNA loaded were: 1 μg unoperated rat sciatic nerve, 5 μg cultured Schwann cells (see Figs. 3 and 6). In experiments that were analyzed by densitometry, 10 μg total RNA from cultured Schwann cells were electrophoresed (see Fig. 4). Ethidium bromide (1 μg/ml) was added to the loading buffer so that the amount of transferred RNA could be visualized on the nylon membrane before hybridization.

**Probes.** The rat p75NGFR cDNA was a 1.3-kb insert isolated from the original construct (38). The rat myelin P₀ cDNA was a 1.9-kb insert isolated from a rat sciatic nerve crush library (7). The rat SCIP cDNA was a 1.5-kb insert containing the 3'-untranslated sequence (32). Composite blots as seen in Figs. 4 and 6 were first probed with the 32P-labeled cDNA insert encoding p75NGFR. After development, blots were stripped by the method described (28) and reprobed with 32P-labeled cDNA encoding myelin P₀. This procedure was repeated before hybridization. All probes were labeled by random priming (GIBCO-BRL, Gaithersburg, MD) and had specific activities of 1–3 × 10⁶ cpm.

**Densitometry.** Densitometric analysis was performed according to the procedures for the LKB Laser Densitometer (LKB Instruments, Bromma, Sweden) and had specific activities of 1–3 × 10⁶ cpm.

**Results**

**Schwann Cell–Neuronal Co-culture Permits Neuronal Influence without Contact**

A co-culture paradigm (Fig. 1) was designed to investigate neuronal regulation of Schwann cell genes. Primary Schwann cells (Fig. 1 c) that have been isolated from adult rat sciatic nerve after a conditioning crush injury and grown to a confluent monolayer, are cultured with E8 chick dorsal root ganglion neurons (Fig. 1 b). The neurons (N) are plated on a microporous membrane a millimeter above the Schwann cells (SC). In this paradigm neurons and Schwann cells communicate through diffusion of ions, proteins or macromolecules but not through cell contact. We confirmed that the 0.4-μm pore does not permit passage of cells or neurites by scanning EM (Fig. 2) and by toluidine blue staining of the underside of the membrane. After 2 d in co-culture, neurons have aggregated and extend fascicles of neurites (Fig. 2 A). No cellular material was observed on the underside of the membrane (Fig. 2 C) which can be compared to the cellular side (Fig. 2 B) at the same magnification. At higher magnifications (10,000) the underside of the membrane was acellular (data not shown). These results are not surprising because neuronal growth cones require substrate attachment to grow (5) and they would not survive extension through the 1 mm of media solution to reach the Schwann cell monolayer.

**Neurons Regulate Schwann Cell p75NGFR mRNA Expression**

Schwann cells present in unoperated sciatic nerve do not express p75NGFR (13). The induction of Schwann cell p75NGFR correlates with loss of axon contact in vivo in sciatic nerve distal to a crush or axotomy (13) and in vitro when Schwann cells are isolated from the nerve for primary culture (16, 24). Initial experiments were based on the assumption that an NGF-dependent population of sensory neurons would be most likely to interact with Schwann cells with which they are in contact in vivo (26). Chick E8 DRG neurons were chosen because of their accessibility. The co-culture paradigm permits an examination of the regulation of Schwann cell p75NGFR expression because E8 DRG neurons that constitutively express this gene (12) are separated by the microporous membrane. Results led us to test the regulatory effect of E8 neural retina cells which while not normally in contact with Schwann cells can be supported by them when transplanted (1). As seen by Northern analysis in Fig. 3, a diminu-
Figure 1. The co-culture paradigm (a) allows neuron–Schwann cell interaction without cell contact. E8 chick dorsal root ganglia neurons, DRG, (N) are plated on a microporous filter 1 mm above a confluent monolayer of adult rat Schwann cells (SC). Neurons aggregate and extend fascicles of neurites (b) after 2 d in co-culture with rat Schwann cells. Adult rat Schwann cells (c) plated below the neurons are photographed through the mesh. Bar, 50 μm.

Figure 2. Neurons cocultured on millicell inserts do not extend processes through the microporous mesh as determined by scanning electron microscopy. After 2 d in co-culture fascicles of neurites have attached to the laminin substrate (A and B). Neurites do not penetrate the membrane as no cellular material is visible on the underside of the filter (C). Bar, 50 μm.
Northern analysis of Schwann cell p75 NGFR mRNA expression in response to neuronal co-culture. Total cellular mRNA isolated from Schwann cells that were cultured alone (lanes 1 and 5) or co-cultured with E8 chick hepatocytes (lane 2) or fibroblasts (lane 3) indicates a constitutive p75 NGFR mRNA expression. A diminished expression is evident in Schwann cells co-cultured with either E8 DRG (lane 4) or neural retina (lane 6). The Schwann cell mRNA was isolated after 2 d in culture. Autoradiographic exposure was for 48 h. Ethidium bromide indicates the amount of RNA transferred to the nylon membrane.

Neurons Induce Expression of Schwann Cell Myelin P0 mRNA

The neuronal control of expression of Schwann cell myelin genes was also investigated. The gene encoding the myelin P0 is expressed exclusively in Schwann cells (22, 23). Its expression is reduced in vivo in response to peripheral nerve injury (9, 46) and in vitro when Schwann cells are cultured (24). Composite Northern analysis as seen in Fig. 4, shows that neuronal (chick E8 DRG neurons) co-culture results in a diminution in Schwann cell p75 NGFR expression (Fig. 4, lanes 3 and 5) and a concomitant induction in Schwann cell myelin P0 expression (Fig. 4, lanes 3 and 5, middle panel). The Schwann cell p75 NGFR expression continues to decline with time in neuronal co-culture. This expression can be compared to that of Schwann cells cultured alone for similar time periods (Fig. 4, lanes 2 and 4). The Schwann cell myelin P0 expression increases with time in co-culture (Fig. 4, lanes 3 and 5). This myelin P0 induction can be compared to very low constitutive levels of expression in Schwann cells cultured alone (Fig. 4, lanes 2 and 4). The densitometric analysis seen in Fig. 5 reveals a consistent decline in Schwann cell expression of p75 NGFR message (○) which diminishes to ~60% (n = 6) at 6 d in co-culture with E8 DRG neurons. Conversely, Schwann cell myelin P0 expression increases 100-fold (●) during the same time period of neuronal co-culture.

Regulatory Activities Are Active in Schwann Cell–Neuronal Co-culture Conditioned Medium

Co-culture conditioned medium (CCM) induced neurite outgrowth from primary chick E8 DRG neurons after 1 or 2 d in culture and this activity was blocked by antisera against NGF (data not shown). The CCM therefore, contains NGF, not a surprising finding since Schwann cells synthesize and secrete NGF (2, 13, 27). The CCM was also transferred to naive Schwann cells that had been isolated from crushed sciatic nerve. Schwann cells that were grown 2 d in CCM did not exhibit a diminution in p75 NGFR or an increase in myelin P0 mRNA expression as compared to Schwann cells grown alone (Fig. 6, lane 5). Because of the possible lability of regulatory activities present in co-cultures, initial regulatory events before myelin gene induction were examined. The induction of SCIP, a POU domain transcription factor (32), has been shown to precede myelin P0 and myelin basic protein induction in development (25, 33) and in regeneration of sciatic nerve (33, 43). Analysis of Schwann cell SCIP mRNA induction in co-culture is shown in the composite Northern analysis in Fig. 6. SCIP mRNA is not expressed by confluent Schwann cells grown alone (Fig. 6, lane 1), but is transiently induced after 1 d in co-culture with DRG neurons (Fig. 6, lane 2). After 2 d in co-culture myelin P0 induction can be compared to very low constitutive levels of expression in Schwann cells cultured alone (Fig. 4, lanes 2 and 4). The densitometric analysis seen in Fig. 5 reveals a consistent decline in Schwann cell expression of p75 NGFR message (○) which diminishes to ~60% (n = 6) at 6 d in co-culture with E8 DRG neurons. Conversely, Schwann cell myelin P0 expression increases 100-fold (●) during the same time period of neuronal co-culture.
Figure 5. Densitometric analysis of $p75_{NGFR}$ mRNA repression and the concomitant induction of myelin $P_0$ mRNA expression in adult Schwann cell co-culture with chick E8 DRG neurons. The relative levels of $p75_{NGFR}$ mRNA are expressed as a percentage of the message that is present in Schwann cells grown alone for the same periods of time (○). The increase in Schwann cell myelin $P_0$ mRNA is expressed as fold increase over basal levels present in Schwann cells grown alone for the same periods of time (●). The data represents six separate Northern analyses. Error bars = ±SEM.

mRNA is induced while SCIP mRNA expression is not detectable (Fig. 6, lane 3). If the CCM from the 2 d neuronal co-culture is transferred to confluent naive Schwann cells for 1 d of culture, an induction of SCIP mRNA is evident (Fig. 6, lane 4). If naive Schwann cells are grown in CCM for longer periods this SCIP mRNA induction is not detectable, $p75_{NGFR}$ mRNA levels remain constant and myelin $P_0$ mRNA is not detectable (Fig. 6, lane 5). Thus, the titers of regulatory activities present in CCM is sufficient to induce neurite outgrowth from DRG neurons and a transcription factor in Schwann cells but not myelin protein gene expression in Schwann cells.

Discussion

The goal of this work was an examination of neuronal influence on adult Schwann cell gene expression. The use of the millicell insert permits an investigation of neuronal-Schwann cell interactions in the absence of cell contact. In this paradigm, neurons and Schwann cells are separated as confirmed by scanning EM. Because of this separation, the requirement of axonal contact for neuronal regulatory mechanisms can be examined. This separation also permits analysis of the expression of the $p75_{NGFR}$ gene in Schwann cells even though it is expressed by both cell types. The expression of the POU domain transcription factor, SCIP, the myelin specific gene $P_0$ and $p75_{NGFR}$ were determined by Northern analysis of Schwann cell RNA isolated after time in neuronal co-culture.

The induction of Schwann cell $p75_{NGFR}$ in sciatic nerve distal to an injury correlates with loss of axonal contact while its subsequent decrease correlates with the regrowth of nerve fibers and reestablishment of axon contact (13, 18, 45). We find that neuronal co-culture without axon-Schwann cell contact also results in a diminished expression of Schwann cell $p75_{NGFR}$ mRNA over time. The mechanism of this regulation is not yet understood. It has been suggested that expression of Schwann cell $p75_{NGFR}$ mRNA diminishes with axonal contact as growth cone high-affinity NGF receptors remove Schwann cell $p75_{NGFR}$-bound NGF (18). This hypothesis implies direct axon-Schwann cell membrane signalling through occupancy of the Schwann cell $p75_{NGFR}$. In our experimental paradigm, Schwann cell-produced NGF is available through diffusion and could not contribute to the diminished $p75_{NGFR}$ mRNA expression through a cell contact mechanism. The sensory neurons present in the coculture express the high-affinity NGF receptor, trk (47), and their internalization of Schwann cell-derived trophic activities that include NGF may be a component of the observed regulation of the Schwann cell $p75_{NGFR}$.

Concomitant with the down regulation of Schwann cell $p75_{NGFR}$ mRNA in neuronal co-culture without cellular contact is the induction of myelin $P_0$ mRNA. The expression of myelin $P_0$ in vivo in response to injury (9) and regeneration (46) is the inverse of $p75_{NGFR}$ mRNA expression. Schwann cells distal to a sciatic nerve crush (20, 21) or in culture (24) do not express myelin genes. Expression of myelin $P_0$ in cultured neonatal Schwann cells is induced by forskolin and other agents that increase intracellular cAMP (21, 34) or by cell-cell contact with neurons from several sources (22, 35). Interestingly, cAMP and its analogues have also been implicated in the down-regulation of $p75_{NGFR}$ (31). These observations in the literature, suggest that cAMP may be a second...
messenger involved in the regulation of adult Schwann cell \(\text{p75}^{\text{NTR}}\) and myelin \(\text{P}_0\) expression in the neuronal co-culture paradigm described here. The opposite effects that neuronal co-culture has on these two genes implies a Schwann cell production of regulatory transcription factor(s).

Some of the diffusible substances involved in the observed gene regulation are labile. When transferred to naïve confluent Schwann cells, CCM does not affect levels of expression of the transcripts of \(\text{p75}^{\text{NTR}}\) or myelin \(\text{P}_0\). Yet, both the action of Schwann cell–derived NGF and an initial regulatory event, the derepression of SCIP, which has been shown to precede myelin \(\text{P}_0\) induction \((25, 32, 33)\), occur in response to CCM. This SCIP mRNA expression induced by CCM is transient as it is its induction after 1 d in co-culture (see Fig. 6, lane 4). SCIP induction may require a relatively low titer of regulatory molecule(s) such as might be necessary for the activation of the cAMP pathway. It is possible that neurons stimulate the Schwann cells to secrete autocrine “regulatory” molecule(s) that influence the derepression of the SCIP gene in the relatively short time period observed for activation, i.e., 24 h. Another possibility is that time is required to achieve an adequate titer of the “regulatory” molecule(s) that would result in a complete activation of the myelin genetic program. Alternatively, a continuous reciprocal exchange and uptake of molecules between neurons and Schwann cells may be necessary. Transfer of CCM would eliminate an essential component, i.e., the neurons. The identity of the diffusible substance(s) is as yet unknown.

The observed regulation is neural specific as other cell types in co-culture have no effect on the expression of these Schwann cell genes. The regulatory capacity is not exclusive to neurons that would normally contact Schwann cells such as DRG or motoneurons. Neurons of the central nervous system, i.e., neural retina, have a similar effect on Schwann cell \(\text{p75}^{\text{NTR}}\) and myelin \(\text{P}_0\) transcript expression. This result is in agreement with studies in which DRG, superior cervical ganglia and stellate ganglia neurons were all capable of inducing myelin \(\text{P}_0\) when their axons were in contact with neonatal Schwann cells in vitro \((35)\).

The regulatory events observed in the co-culture paradigm mimic what has been observed during regeneration in the adult. The Schwann cell gene regulation described is reminiscent of developmental events. During neural crest migration and peripheral nerve development, neurons in contact with glial precursors direct Schwann cell differentiation \((16, 17, 48)\), and myelin gene induction \((29, 48)\). SCIP has been shown to be expressed in both the central and peripheral nervous systems before the induction of myelin genes and the subsequent expression of myelin proteins \((22, 33; S. Scherer, submitted for publication). These other studies summarized above using cells or tissues in culture \((39–42)\) or the crushed rat sciatic nerve model have provided abundant evidence that the regulation of myelination and of myelin specific genes in Schwann cells and Schwann cell proliferation during Wallerian degeneration result from axon contact. The data reported here demonstrate that adult Schwann cells are also susceptible to diffusible neuronal signals. How much of the myelination program can be reiterated in Schwann cells by these signals as compared to cell–cell contact signals has not yet been determined. The data presented here demonstrate neuronal regulation of three Schwann cell genes. Other myelin and cell adhesion proteins may be under similar control. The ability of neurons to direct an adult Schwann cell genetic program at a distance suggests a possible mechanism by which regenerating axons can initiate myelination in advance of the growth cone.

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