Glial Growth Factor/Neuregulin Inhibits Schwann Cell Myelination and Induces Demyelination

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Abstract. During development, neuregulin-1 promotes Schwann cell proliferation and survival; its role in later events of Schwann cell differentiation, including myelination, is poorly understood. Accordingly, we have examined the effects of neuregulin-1 on myelination in neuron-Schwann cell cocultures. Glial growth factor (GGF), a neuregulin-1 isoform, significantly inhibited myelination by preventing axonal segregation and ensheathment. Basal lamina formation was not affected. Treatment of established myelinated cultures with GGF resulted in striking demyelination that frequently began at the paranodes and progressed to the internode. Demyelination was dose dependent and accompanied by dedifferentiation of Schwann cells to a promyelinating stage, as evidenced by reexpression of the transcription factor suppressed cAMP-inducible POU; a significant proportion of cells with extensive demyelination also proliferated. Two other Schwann cell mitogens, fibroblast growth factor-2 and transforming growth factor-β, inhibited myelination but did not cause demyelination, suggesting this effect is specific to the neuregulins. The neuregulin receptor proteins, erbB2 and erbB3, are expressed on ensheathing and myelinating Schwann cells and rapidly phosphorylated with GGF treatment. GGF treatment of myelinating cultures also induced phosphorylation of phosphatidyl-inositol 3-kinase, mitogen-activated protein kinase, and a 120-kD protein. These results suggest that neuronal mitogens, including the neuregulins, may inhibit myelination during development and that activation of mitogen signaling pathways may contribute to the initial demyelination and subsequent Schwann cell proliferation observed in various pathologic conditions.

Key words: Schwann cell • demyelination • mitogen • neuregulin • erbB

Introduction

Schwann cell generation and differentiation are tightly regulated by axons. During development, axons promote Schwann cell proliferation via contact-dependent mitogen(s) (Salzer et al., 1980); competition for limiting quantities of neuron-derived trophic factors ensures that the number of Schwann cells is matched to the number and length of axons (reviewed in Jessen and Mirsky, 1998). Once a full complement of Schwann cells is generated, axons together with the extracellular matrix promote Schwann cell differentiation, regulating whether they ensheathe or myelinate axons (reviewed in Bunge et al., 1986). Conversely, axotomy initiates a characteristic series of dramatic events in the distal stump, collectively referred to as Wallerian degeneration, that includes the degeneration of axons and myelin sheaths and the dedifferentiation and proliferation of Schwann cells (Scherer and Salzer, 1996). While the molecular cues that regulate

Schwann cell generation have begun to emerge, the signals that regulate Schwann cell differentiation and their response to axotomy remain poorly understood.

Axons promote the generation of Schwann cells, in large part, via the activity of the neuregulins (reviewed in Adlkofer and Lai, 2000). The neuregulins are a family of alternatively spliced, soluble and membrane-bound proteins encoded by four known genes. Neuregulin-1, which was the first member of this family to be described and remains the best characterized, is highly expressed by peripheral nervous system and central nervous system neurons during development (Garratt et al., 2000a). Studies with the soluble neuregulin-1 isoform, glial growth factor (GGF),[1] indicate that this gene family can restrict neural crest cells to the glial lineage (Shah et al., 1994) and promote the maturation, proliferation, and survival of Schwann cell precursors (Dong et al., 1995). Similarly, neuregulin-1

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[1]*Abbreviations used in this paper:* DRG, dorsal root ganglia; GGF, glial growth factor; MAP, mitogen-activated protein; MBP, myelin basic protein; PI, phosphatidylinositol; SCIP, suppressed cAMP-inducible POU.

promotes the proliferation (Morrissey et al., 1995) and survival (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996) of committed Schwann cells associated with axons. In striking confirmation of its importance in Schwann cell development, mice with targeted disruptions of the neuregulin-1 gene (Meyer and Birchmeier, 1995), or a major isoform expressed on peripheral nerves (Wolpowitz et al., 2000), exhibit a severe deficiency of precursors or of committed Schwann cells, respectively.

Neuregulin-1 isoforms mediate their effects by binding to, and activating, members of the erbB receptor tyrosine kinase family, which includes the EGF receptor (erbB1), erbB2, erbB3, and erbB4 (reviewed in Adlkofer and Lai, 2000). Ligand binding is believed to result in receptor dimerization, tyrosine phosphorylation, and subsequent activation of intracellular signaling pathways (reviewed in Burden and Yarden, 1997). ErbB2 and erbB3 are the major receptor proteins expressed by Schwann cells and their precursors during peripheral nerve development (Cohen et al., 1992; Shah et al., 1994; Levi et al., 1995). Consistent with their critical role, Schwann cells fail to develop in peripheral nerves of mice with knockouts of either erbB2 or erbB3 (for a recent review, see Garratt et al., 2000a).

While the role of neuregulin-1 in early Schwann cell development is well documented, its role in later events of Schwann cell differentiation, including myelination, is less clear. Neuregulin-1 continues to be expressed at significant levels by neurons in the adult nervous system (Chen et al., 1994), including motor and large sensory neurons (Bermingham-McDonogh et al., 1997). These findings are consistent with a functional role in the adult that includes maintenance of neuromuscular junctions (Sandrock et al., 1997) and, possibly, of myelin sheaths. Indeed, recent studies in which the erbB2 gene was conditionally ablated in developing Schwann cells provide evidence that this neuregulin receptor subunit is important for myelination (Garratt et al., 2000b). In potential agreement, neurons activate phosphatidylinositol (PI) 3-kinase in Schwann cells via neuregulin-1 and this activity, in turn, is required for the generation of Schwann cells and the early events of myelination (Maurel and Salzer, 2000). A potential limitation of both studies is that they do not distinguish between a role of neuregulin signaling in the initial axon–Schwann cell interactions required for subsequent events of myelination versus a direct effect on myelination itself.

Accordingly, we have examined the effects of the neuregulin-1 isoform, GGF, on the differentiation and myelination of rat Schwann cells cultured with dorsal root ganglion neurons. We now report that addition of GGF to such cultures potently blocks myelination in a dose-dependent manner. Addition of GGF to cultures in which myelin had already formed results in striking demyelination associated with Schwann cell dedifferentiation and proliferation. ErbB2 and erbB3 are rapidly phosphorylated and PI 3-kinase and mitogen-activated protein (MAP) kinase are rapidly activated at the onset of GGF-induced demyelination. These results suggest a novel mechanism of demyelination in which activation of mitogen signaling pathways by neuregulin-1, and possibly other mitogens, leads to myelin breakdown and Schwann cell dedifferentiation.

Materials and Methods

Antibodies and Immunofluorescence

Mouse mAb included the anti–myelin-associated glycoprotein MA 513 (gift of J. Roder, Mt. Sinai Hospital, Toronto, Ontario, Canada), anti–myelin basic protein (MBP) SMI 94 (Sternberger Monoclonals, Inc.), anti–phosphotyrosine RC20 (Transduction Laboratories, Inc.), and anti-BrdU 1202693 (Roche Molecular Biochemicals). Rabbit polyclonal antibodies included anti–MBP (gift of D. Colman, Mt. Sinai Medical Center, New York, NY), antilaminin L-9393 (Sigma-Aldrich), anti–erbB2 sc-284, anti–erbB3 sc-285 (Santa Cruz Biotechnology, Inc.), and anti–suppressed cAMP-inducible POU (SCIP) 3-145 (gift of G. Lemke, The Salk Institute, La Jolla, CA). Secondary antibodies included affinity-purified, rhodamine-conjugated donkey anti–rabbit IgG, fluorescein-conjugated donkey anti–mouse IgG (Chemicon International, Inc.) and HRP-conjugated goat anti–rabbit IgG (Amersham Pharmacia Biotech). Cultures were processed for immunofluorescence microscopy as described previously (Einheber et al., 1995).

To quantitate the extent of myelination, the total number of myelin sheaths was counted in each of six random 40× fields per coverslip (12 fields per condition per experiment, total of two experiments). To quantitate the extent of demyelination, the total numbers of normal and damaged myelin sheaths were counted in each of six random 40× fields per coverslip (12 fields per condition per experiment, total of two experiments). Myelin sheaths were classified as damaged if they contained vesicles or if the sheaths were crenated or split.

Cell Culture

Rat Schwann cells and embryonic day 16 dorsal root ganglion (DRG) neurons were isolated as described (Einheber et al., 1995). Neurons were plated in C media, consisting of MEM (Life Technologies), 10% FBS (Hy-Clone Laboratories), 4 mg/ml glucose (Sigma-Aldrich), 2 mM glutamine (Life Technologies), and 50 ng/ml NGF (Harlan Bioproducts for Science). Cultures were cycled on C media supplemented with 5-fluorodeoxyuridine and uridine (Sigma-Aldrich), both at 10^{-5} M, every other feeding for 2.5 wk to remove nonneuronal cells. Schwann cell-neuron cocultures were prepared by adding 200,000 Schwann cells to each coverslip of purified neurons. The next day, the media was replaced with N2 media (Bottenstein and Sato, 1979) supplemented with 50 ng/ml NGF. The cocultures were kept in N2 media for 3 d to allow the Schwann cells to repopulate the neurites. To initiate basal lamina formation and myelination, some of the cocultures were treated for 3–4 wk with C media supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich). To generate premyelinated cocultures, other cocultures were switched from N2 media to C media and maintained in this media for 3–4 wk.

Electron Microscopy

In some cases, cultures treated with GGF were processed for and analyzed by electron microscopy. DRG neurons were cultured on Aclar (Allied Signal) plastic coverslips for 2.5 wk, seeded with Schwann cells, and switched to N2 media for 3 d. Some of the cocultures were then treated with C media supplemented with ascorbic acid with or without 200 ng/ml GGF and maintained for 7 d. Other cocultures were treated with C media supplemented with ascorbic acid for 21 d to allow myelination to occur. These cocultures were then treated for 3 d with C media supplemented with ascorbic acid with or without 200 ng/ml GGF. All of the cocultures were fixed, embedded in Epon-araldite and processed for electron microscopy as described (Einheber et al., 1995).

Proliferation Assays

To determine the relationship between Schwann cell proliferation and demyelination in Schwann cell-DRG neuron cocultures, myelinated cocultures were treated with 200 ng/ml GGF for 36 or 48 h in the continuous presence of 20 μM BrdU. The cocultures were fixed and stained for MBP and fixed again and processed for anti–BrdU labeling as previously described (Maurel and Salzer, 2000). Six random fields were photographed per coverslip (12 fields per condition per experiment, total of two experiments); BrdU- and Hoechst-labeled nuclei in each field were counted. All of the myelin sheaths in each field were also counted and characterized as either "not demyelinated," "moderately demyelinated" if up to half of the myelin sheath was damaged, or "extensively demyelinated" if more than half of the myelin sheath was damaged. A Schwann cell nucleus was considered to be associ-
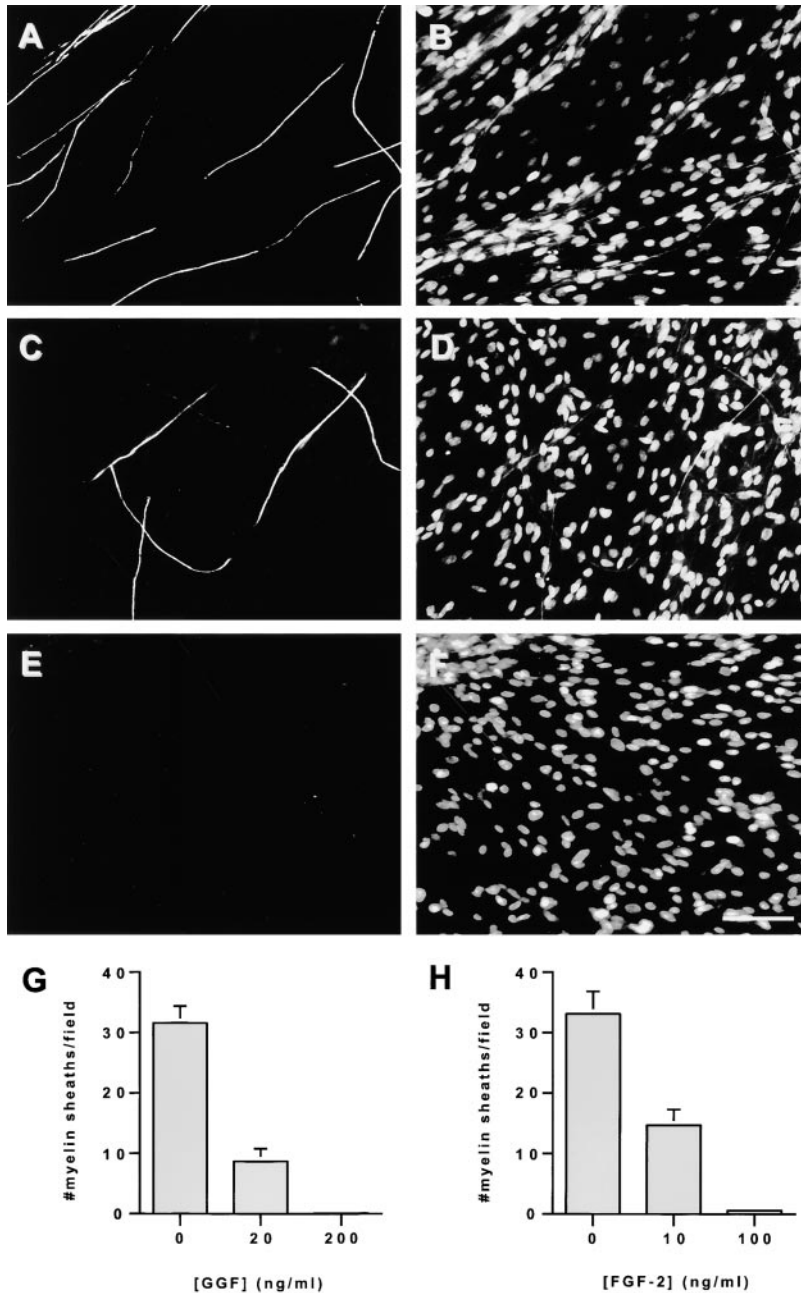
Figure 1. GGF and FGF-2 inhibit myelination. (A–F) Schwann cell-neuron cocultures were grown in myelin-promoting media without (A and B) or with 20 (C and D) or 200 (E and F) ng/ml GGF and maintained for 7 d. The cocultures were fixed and stained for MBP (A, C, and E) and Hoechst dye to visualize Schwann cell nuclei (B, D, and F). GGF strikingly inhibited myelination in a dose-dependent manner. Bar, 100 μm. (G and H) Schwann cell-DRG neuron cocultures were switched to myelin-promoting media with different concentrations of GGF (G) or FGF-2 (H) and maintained for 10 d. Cultures were fixed and stained for MBP and the numbers of myelin sheaths per high-powered field were counted. The mean values and SEM for each GGF and FGF-2 concentration from two representative experiments are shown. The number of myelin sheaths in the cultures treated with either GGF or FGF-2 was significantly lower than that in control cultures ($P \leq 0.005$).

ated with an internode if it directly abutted a myelin sheath. Student's *t* tests were performed with the Statview software package (SAS Institute, Inc.).

Western Blots

ErbB2 and erbB3 levels, and the effects of GGF on erbB phosphorylation, were determined by Western blot analysis as described (Canoll et al., 1996). In brief, cultures of Schwann cells, neurons, premyelinated, and myelinated cocultures were lysed after treating with or without 200 ng/ml GGF for 2.5 min at 37°C, in chilled lysis buffer (Canoll et al., 1996). Similarly, myelinated cocultures were lysed directly or after treatment with 20 or 200 ng/ml GGF for 3 d. Protein concentrations were determined by the Micro BCA method (Pierce Chemical Co.). 15 μg of protein were fractionated by SDS-PAGE, electroblotted onto nitrocellulose, and probed with anti–erbB2, anti–erbB3 or antiphosphotyrosine antibodies. Proteins were visualized by addition of HRP goat anti–rabbit Ig and enhanced chemiluminescence reagents according to the manufacturer's instructions (Amersham Pharmacia Biotech).

The time course of GGF-induced tyrosine phosphorylation was analyzed by treating myelinated cocultures with 200 ng/ml GGF in C media with ascorbic acid for 0, 1, 2.5, 5, 10, and 30 min at 37°C. The cocultures were lysed and processed for Western blotting as described above.

Kinase Assays

Myelinated cocultures were maintained in N2 media for 15 h, and then treated with or without 200 ng/ml GGF for 10 min at 37°C. Immunoprecipitation and measurement of MAP kinase and PI 3-kinase activities were performed as described previously (Canoll et al., 1999).

Results

GGF and FGF-2 Inhibit Myelination

To determine whether neuregulin-1 regulates Schwann cell differentiation, we examined the effects of exogenous GGF on myelination in neuron-Schwann cell cocultures. Cocultures were maintained for 1 wk or longer in media contain-
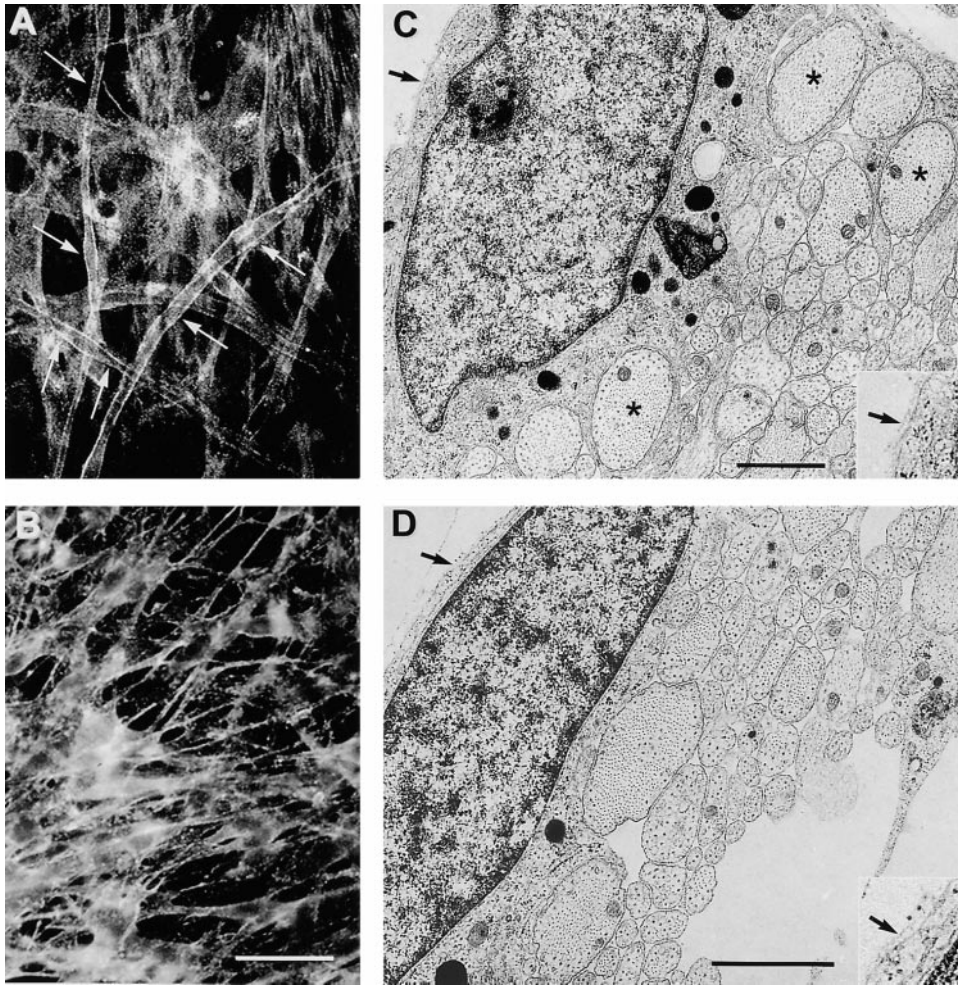
Figure 2. GGF inhibits ensheathment but not basal lamina formation. Cocultures were maintained in myelin-promoting media without (A and C) or with 200 ng/ml GGF (B and D). One set of cultures was fixed after 11 d and laminin expression was visualized by immunofluorescence (A and B). In control cocultures, large axons have formed an apparent 1:1 relationship with Schwann cells and are surrounded by basal lamina tubes (arrows); such laminin tubes are absent in the treated cultures. A second set of cultures was fixed after 1 wk and analyzed by electron microscopy (C and D). In the control cultures, several axons (*) are segregated by Schwann cell processes, whereas, in the GGF-treated cultures, axons were unensheathed despite the presence of a normal basal lamina (arrow). Bar, 50 μm (A and B) and 0.2 μm (C and D).

ing serum and ascorbate to promote myelination with or without the addition of 20 or 200 ng/ml of GGF (corresponding to ~0.25 and 2.5 nM, respectively). Cultures were fixed and myelin sheaths were visualized by staining for MBP (Fig. 1). Results demonstrate that GGF strikingly inhibited myelination in a dose-dependent manner. Micrographs of representative cocultures demonstrating MBP expression (Fig. 1, A, C, and E) and the nuclei from corresponding fields (B, D, and F) are shown. Control cultures (Fig. 1, A and B) demonstrated numerous myelin sheaths; cultures treated with 20 ng/ml (C and D) or 200 ng/ml (E and F) of GGF demonstrated substantial (>50%) or essentially complete inhibition of myelination, respectively. These results are quantitated in Fig. 1 G. This inhibition of myelination was contingent upon continued GGF treatment. Cultures treated for 21 d continued to demonstrate partial or complete inhibition of myelination with 20 or 200 ng/ml of GGF; removal of GGF from the media at any point led to normal myelination (data not shown). These results indicate that GGF reversibly inhibits myelination.

We have previously demonstrated that TGF-β1, which is mitogenic for isolated Schwann cells, also inhibits myelination in vitro (Einheber et al., 1995). To examine whether other Schwann cell mitogens inhibit myelination, we performed a similar analysis with FGF-2. Fig. 1 H reveals that FGF-2 was an equally potent inhibitor of myelination. Addition of 10 ng/ml (corresponding to ~0.5 nM)

partially inhibited and 100 ng/ml of FGF-2 essentially completely blocked myelin formation. Taken together, these data demonstrate that a variety of Schwann cell mitogens inhibit myelination.

To investigate the mechanism by which GGF blocks myelination, we examined basal lamina formation and the ultrastructure of treated and control cultures (Fig. 2). Basal lamina formation appeared to be unaffected based on the intensity of laminin staining (Fig. 2, A and B). There was a striking change in the organization of the cultures, however. In control cultures, the basal lamina frequently surrounded Schwann cells that were longitudinally oriented in an apparent 1:1 relationship with an axon (Fig. 2 A, arrows). In contrast, Schwann cells in the treated cultures apparently failed to form a 1:1 relationship with axons despite expressing significant amounts of laminin at their surfaces (Fig. 2 B). EM analysis confirmed that GGF inhibited the segregation of neurites by Schwann cells. Fig. 2 C shows a control culture in which axons (*) were ensheathed by Schwann cell processes; in other fields, axons were in the early stages of myelination. In contrast, in the GGF-treated cultures, axonal ensheathment was limited and fibers were rarely segregated in a 1:1 relationship (Fig. 2 D). In both control and treated cultures, a well-formed basal lamina was present at the abaxonal Schwann cell surface, consistent with the laminin staining (Fig. 2, C and D, arrows). These results indicate that the failure of Schwann
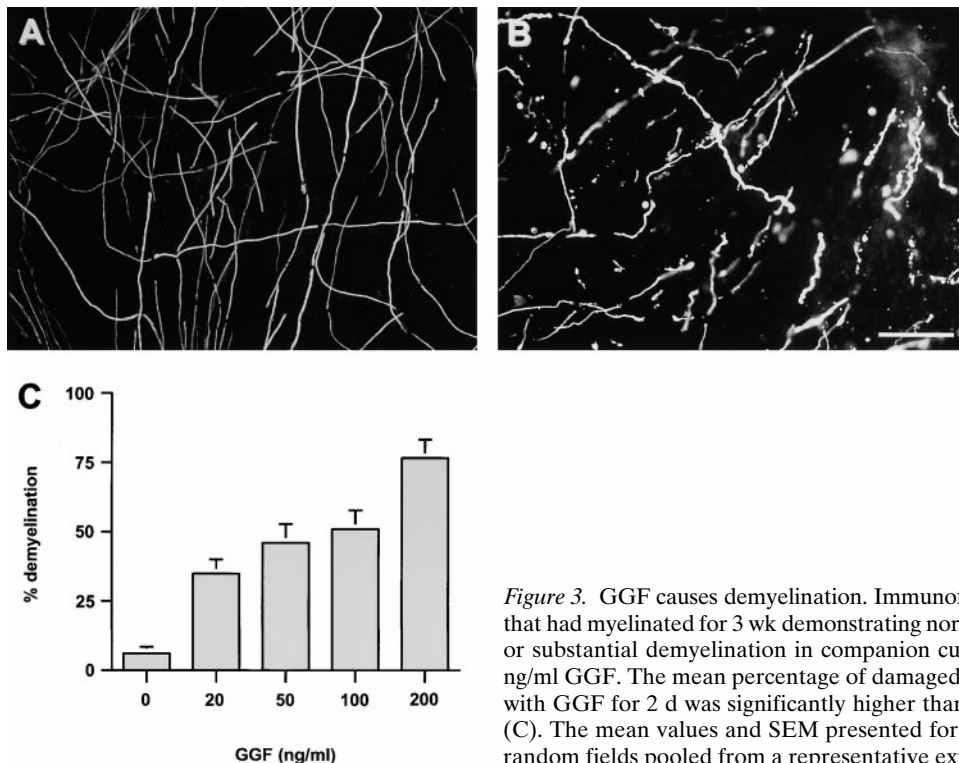
Figure 3. GGF causes demyelination. Immunofluorescence micrographs of cocultures that had myelinated for 3 wk demonstrating normal myelination in control cultures (A) or substantial demyelination in companion cultures after 3 d of treatment with 200 ng/ml GGF. The mean percentage of damaged myelin sheaths in the cultures treated with GGF for 2 d was significantly higher than that in control cultures ($P < 0.0001$) (C). The mean values and SEM presented for each GGF concentration are from 12 random fields pooled from a representative experiment. Bar, 100 μm.

cells to ensheathe axons did not result from an effect of GGF on basal lamina formation.

GGF Induces Demyelination

We next analyzed the effects of GGF on cocultures in which myelin had already formed. Treatment of established, myelinated cocultures with GGF for 2 or 3 d resulted in striking demyelination. Fig. 3 A shows an immunofluorescence micrograph of a representative field from a myelinated coculture, and B shows another coculture undergoing substantial demyelination after 3 d of continuous treatment with 200 ng/ml of GGF. The demyelinating effects of GGF were dose dependent (Fig. 3 C). Significant demyelination was observed with 2 d of treatment even at 20 ng/ml; more extensive demyelination was evident at higher concentrations. At GGF concentrations of 200 ng/ml, ~75% of the myelin sheaths degenerated; the remaining sheaths displayed no obvious morphological defects. While this effect was most robust with continuous treatment, nearly comparable demyelination was observed after only 30 min of GGF treatment followed by its removal (data not shown). Finally, treatment with either FGF-2 (10 and 100 ng/ml) or TGF-β1 (10 and 100 ng/ml) did not induce obvious myelin sheath degeneration, indicating this effect was specific to the neuregulins (data not shown).

Fibers undergoing demyelination were initially characterized by crenated myelin sheaths followed by overt myelin breakdown and ovoid formation. Degeneration of some myelin sheaths was already apparent after 24 h of treatment, but in most cases maximal demyelination occurred between 36 and 48 h of treatment. Myelin debris was then rapidly cleared and, by 72 h, was substantially absent from the cultures. Demyelination often began at one end of the myelin sheath, progressing from the paranodal region to the Schwann cell internode (data not shown). To analyze this effect further, control and treated cultures were examined by electron microscopy (Fig. 4). In general, we observed significant amounts of myelin debris within myelin segments. Some of these appeared to be present within vacuoles; they were present both in the internode and the paranodal region (Fig. 4 C). These results suggest that Schwann cells are able to clear segments of the myelin sheath while other regions remain remarkably intact.

Demyelination appeared to be a direct, nontoxic effect of GGF on the Schwann cells. Axons associated with degenerating myelin sheaths were intact as indicated by continuous neurofilament immunoreactivity (data not shown). In addition, GGF treatment did not increase Schwann cell or neuron apoptosis based on TdT-mediated dUTP nick-end labeling analysis (data not shown). Consistent with the lack of toxicity, the effects of GGF were reversible, with cocultures remyelinating efficiently even after 1 mo of continuous GGF treatment was discontinued (data not shown). Taken together, these studies indicate that the demyelination resulting from GGF treatment is specific, reversible, and likely to be a direct effect on the Schwann cell.

GGF Promotes the Dedifferentiation of Myelinating Schwann Cells

Previous studies have shown that GGF induces oligodendrocyte progenitors that have differentiated in culture to downregulate their expression of MBP and revert to an earlier stage in their lineage (Canoll et al., 1999). To determine whether GGF similarly induces Schwann cell dedifferentiation, we analyzed its effects on the expression of SCIP, a POU-domain transcription factor that is upregulated in vivo at the promyelinating stage and downregulated during myelination (Zorick et al., 1996; Arroyo et al.,
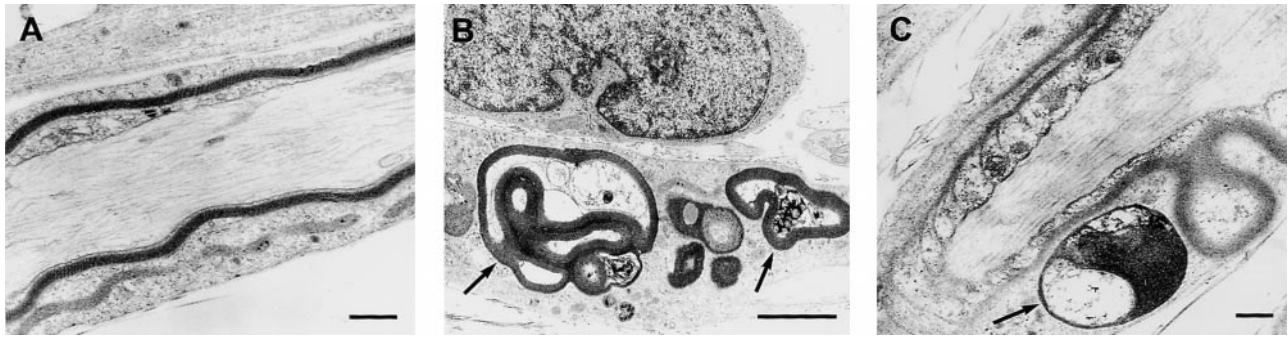
Figure 4. Ultrastructure of control and GGF-treated cocultures. Schwann cell-DRG neuron cocultures that had myelinated for 3 wk were maintained for an additional 3 d without (A) or with (B and C) 200 ng/ml GGF. The cocultures were then fixed and processed for electron microscopy. (A) Longitudinal section through a control internode, (B) sections of two Schwann cells; note the presence of numerous myelin inclusions (arrows). (C) Section through a paranode that contains a myelin inclusion (arrow) and an abnormal folding of the myelin sheath. Bars, 0.2 μm.

1998). Consistent with previous findings in vivo, SCIP was expressed at high levels in the nuclei of Schwann cells before myelination (Fig. 5 A) and was substantially downregulated in the nuclei of mature, myelinating Schwann cells (outlined in A and B). When GGF was added to myelinated cocultures for 3 d, there was a dramatic increase in SCIP expression in myelin-related Schwann cells, including those associated with apparently intact as well as with degenerating myelin sheaths (outlined in C and D). These results indicate that GGF induces myelinated Schwann cells to dedifferentiate to the promyelinating stage.

GGF-induced Demyelination Does Not Require Proliferation

To determine whether GGF inhibited myelination, or promoted demyelination, by first inducing Schwann cell proliferation, we incubated cocultures with BrdU and GGF.

We first asked whether GGF had any effect on the rate of proliferation during early stages of myelination, when some Schwann cells were still dividing in association with axons. GGF did not further increase the labeling index of Schwann cells proliferating in association with neurons, although it was mitogenic for isolated Schwann cells included as a positive control (data not shown). Thus, proliferation was ~20% after 1 d and 10% after 3 d of coculture with neurons, with or without supplemental GGF. These results suggest that neuregulin levels do not limit the proliferation rate of Schwann cells associated with neurites.

We next examined whether GGF could induce the proliferation of post-mitotic, myelinating Schwann cells. To this end, cultures that had been myelinating for 3 wk were continuously treated with GGF for either 36 or 48 h in the presence of BrdU; cultures were then double stained for BrdU and MBP to identify myelinating or previously my-
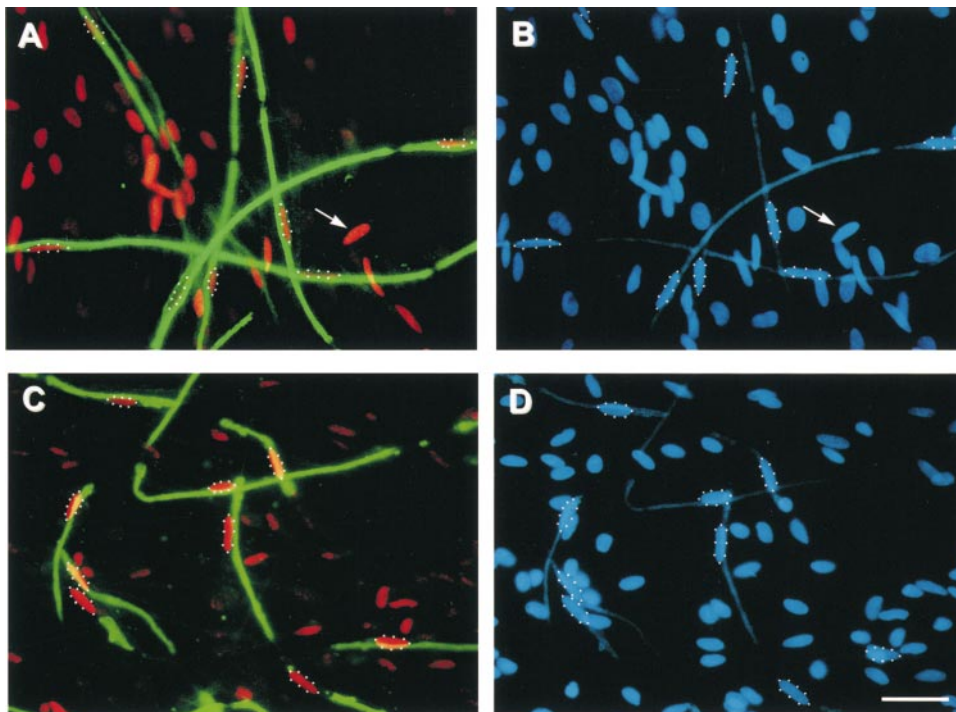

Figure 5. GGF causes dedifferentiation of myelinating Schwann cells. Cocultures that had myelinated for 3 wk were maintained for an additional 3 d without (A and B) or with (C and D) 200 ng/ml GGF. Cocultures were then fixed and stained for SCIP (red) and MBP (green) (A and C); staining of nuclei of the same fields is also shown (B and D). GGF treatment significantly increased SCIP expression in Schwann cell nuclei associated with myelin sheaths, several of which exhibit partial loss of myelin at one or both ends. The nuclei of myelinating Schwann cells are outlined; a premyelinating Schwann cell nucleus in the control culture is indicated with an arrow (A). Bar, 50 μm.

GGF	Schwann cell	Cells with intact myelin sheaths	Cells with moderate degeneration	Cells with extensive degeneration
ng/ml				
0	1.4 ± 0.4	0	0	0
200	6.4 ± 0.8*	3.0 ± 2.1	2.8 ± 2.8	27 ± 8.1*

Myelinated cocultures were incubated with BrdU for 36 h in media with or without 200 ng/ml GGF. The cocultures were then fixed and stained for BrdU, MBP, and Hoechst. *Proliferation was increased significantly the GGF-treated cultures for the total population os Schwann cells ($P < 0.0001$) and for cells with myelin sheaths. Analysis of MBP⁺ Schwann cells indicated that proliferation was significantly increased in cells with sheaths undergoing extensive (>50%) degeneration ($P < 0.005$) in the GGF-treated cultures. Cells with moderate degeneration (i.e., <50%) also showed increased proliferation that did not reach statistical significance. The mean values and SEM presented for each GGF concentration are from 12 random fields pooled from a representative experiment.

elinated cells. In the case of MBP^{+} cells, we further distinguished between cells with an intact myelin sheath and those with either moderate or advanced demyelination. Results are summarized in Table I.

GGF induced a significant, approximately fourfold increase in the overall rate of Schwann cell proliferation, which is normally quite low in these mature cultures (i.e., from 1.4 to 6.4% after 36 h of BrdU incorporation). When MBP^{+} Schwann cells were analyzed, the mitogenic effects of GGF depended on the extent of demyelination. A small percentage of Schwann cells with apparently intact myelin sheaths were BrdU[+] in the treated cultures. As myelinating Schwann cells normally do not proliferate (Salzer and Bunge, 1980; see also Fig. 6 A), a labeling index of 3% in such cells is significant. These results indicate that proliferation of myelinating Schwann cells can occur in the absence of obvious demyelination. Cells with myelin sheaths in late stages of degeneration proliferated the most extensively in response to GGF treatment, exhibiting a labeling index of 27%; several examples are shown in Fig. 6 C.

Strikingly, most Schwann cells, even those with significant myelin degeneration, did not proliferate during the 36-h treatment period; thus, 97% of cells with moderate and 73% of those with advanced myelin sheath degeneration remained unlabeled (Table I). The latter results indicate that GGF-induced demyelination does not require Schwann cells to re-enter the cell cycle. After 48 h of GGF treatment, demyelination was more extensive and the labeling index increased to ~50% in those cells with extensive myelin sheath degeneration. Even at this later time point, however, many Schwann cells with substantial demyelination were unlabeled. Taken together, these studies indicate that demyelination does not require Schwann cell proliferation and that proliferation typically occurs when demyelination is quite advanced.

Phosphorylation of GGF Receptors and Activation of Signaling Pathways

Finally, we characterized the effects of GGF on neuregulin receptors and downstream signaling pathways. Detergent lysates were prepared directly from Schwann cell, DRG neuron, premyelinated, and myelinated cocultures or after treating these cultures with 200 ng/ml of GGF for 2.5 min. Lysates were fractionated by SDS-PAGE, blotted onto nitrocellulose, and probed with anti–erbB2, anti–erbB3, or antiphosphotyrosine antibodies. As previously reported (Levi et al., 1995; Canoll et al., 1996), erbB2 and erbB3 were robustly expressed in Schwann cells (Fig. 7 A). GGF increased the tyrosine phosphorylation of p185, which corresponds to the expected mol wt of erbB2 and erbB3 (Fig. 7 A). In contrast, minimal erbB2 and erbB3 were detected by Western blotting of neurons, although low-level erbB2 expression was observed by immunofluorescence (data not shown). Consistent with this limited erbB expression, no phosphorylation of p185 was observed after GGF treatment of the DRG neuron cultures.
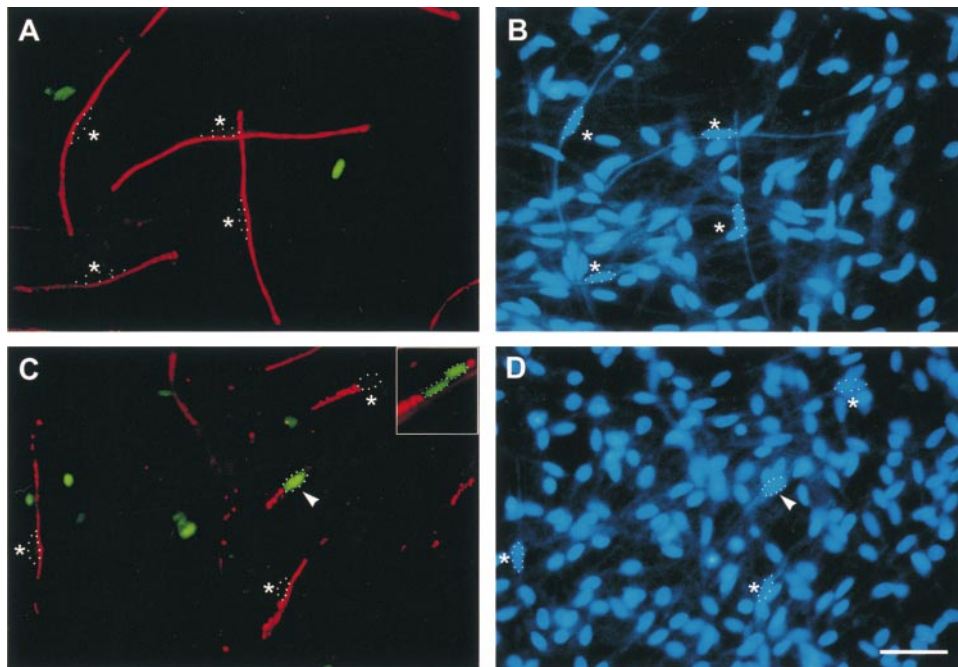

Figure 6. Demyelination and proliferation are not obligately linked. Myelinated cocultures were incubated with BrdU for 36 h in media without (A and B) or with (C and D) 200 ng/ml GGF. The cocultures were then fixed and stained for BrdU (green) and MBP (red); nuclei are stained with Hoechst (blue). In control cultures, myelinating Schwann cells do not incorporate BrdU (four representative nuclei are encircled in A and B), whereas BrdU-positive nuclei are relatively common in GGF-treated cultures, typically in cells undergoing extensive demyelination (C, arrowhead and inset). Note also that the nuclei of several degenerating myelin sheaths are BrdU[−] (*). This increase in BrdU labeling was associated with actual Schwann cell mitosis as BrdU[+] nuclei were also present as doublets (C, inset). Bar, 40 μm.
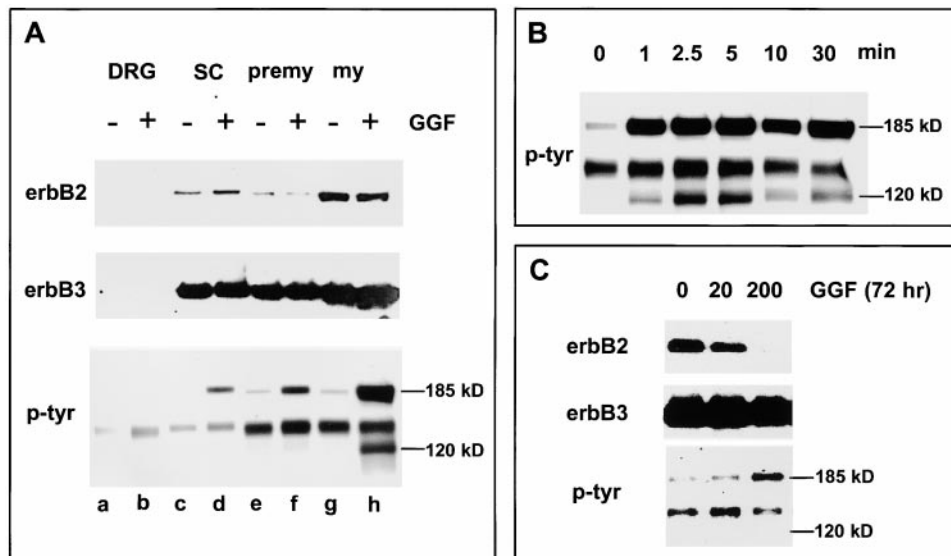
Figure 7. Expression and tyrosine phosphorylation of GGF receptors. (A) Expression and phosphorylation of GGF receptors. DRG neurons, Schwann cells, premyelinated (premy), and myelinated (my) cocultures were lysed directly (−) or after 2.5 min of 200 ng/ml GGF treatment (+). 15 μg of each lysate was fractionated by SDS-PAGE, blotted onto nitrocellulose, and probed with anti–erbB2, anti–erbB3, or antiphosphotyrosine antibodies. ErbB2 and erbB3 were expressed by Schwann cells (c and d) and in the cocultures (e–h). Note that while GGF activated p185 in all cultures containing Schwann cells, p120 was only activated in the myelinated cocultures (h); a constitutively phosphorylated band of ~140 kD was present in all the lanes. (B) Time course of phosphorylation. Myelinated cocultures were lysed either directly or at varying times after treatment with 200 ng/ml GGF. Lysates were fractionated and blotted as described in A, and probed with antiphosphotyrosine antibodies. Both p185 and p120 were rapidly tyrosine phosphorylated and continued to be phosphorylated after 30 min of GGF treatment. (C) Expression and phosphorylation of GGF receptors in demyelinating cocultures. Myelinated cocultures were maintained for 3 d in media without or with 20 or 200 ng/ml GGF. Protein lysates were prepared, fractionated, blotted, and probed as described in A. While there was a dose-dependent decrease in erbB2 (top), erbB3 levels were unchanged (middle). (Bottom) There was a dose-dependent increase in p185 tyrosine phosphorylation in the cocultures treated with GGF for 3 d.

In cocultures of neurons and Schwann cells, erbB2 and erbB3 continued to be expressed at substantial levels in both premyelinating (i.e., cultures maintained in the absence of ascorbate to prevent basal lamina formation) and myelinating cocultures (Fig. 7 A). As the myelinated cultures are heterogeneous, this high level expression of erbB2 and erbB3 may reflect expression by ensheathing Schwann cells, by myelinating Schwann cells, or both. Basal levels of p185 phosphorylation were observed in the premyelinated and myelinated cocultures, but not in Schwann cells alone; addition of GGF substantially increased the phosphorylation of p185 in all Schwann cell culture conditions (Fig. 7 A). Of interest, GGF also induced the tyrosine phosphorylation of a 120-kD protein (p120) in myelinated cocultures, but not in Schwann cells or premyelinating cocultures (Fig. 7 A). Fig. 7 B reveals that p185 phosphorylation increased to near maximal levels after 1 min of GGF treatment; this level was sustained for at least 30 min of GGF treatment. In contrast, p120 phosphorylation, although present at 1 min of GGF treatment, reached maximum levels only after 2.5 min and declined at incubation times of 10 min or longer. These results suggest that p120 phosphorylation is downstream of erbB phosphorylation. Preliminary analysis suggested that p120 is distinct from focal adhesion kinase, c-cbl, and Pyk-2, candidate proteins of similar mol wt (data not shown).

We next analyzed neuregulin receptor expression and phosphorylation in myelinating cocultures continuously treated with GGF. Fig. 7 C demonstrates that erbB3 levels remain constant, whereas erbB2 levels decreased in a dose-dependent fashion, although it remained detectable even in cultures treated with 200 ng/ml of GGF. Surprisingly, p185 continued to be phosphorylated at high levels despite the low levels of erbB2; in contrast, phosphorylation of p120 was no longer detectable after 3 d of GGF treatment.

These results indicate that GGF-induced demyelination is associated with continued erbB phosphorylation.

To investigate the intracellular signaling pathways that may mediate GGF-induced demyelination, we examined whether GGF activates PI 3-kinase and MAP kinase in myelinated cocultures. Myelinating cocultures were lysed directly or after treatment with 200 ng/ml of GGF for 10 min. Immunoprecipitates were prepared with antiphosphotyrosine or anti–extracellular signal-regulated kinase-1 and -2 (erk-1 and erk-2) antibodies, and kinase reactions were performed. GGF induced a 1.5–2-fold activation of MAP kinase and a 5–6-fold activation of PI 3-kinase (data not shown). This robust activation of PI 3-kinase suggests a potential role in mediating GGF-induced demyelination.

Discussion

While neuregulin-1 has a crucial role in peripheral nervous system development (Adlkofer and Lai, 2000), its role, and that of other growth factors, in the later events of peripheral nerve development remains poorly understood. In this study, we have demonstrated that GGF, a soluble neuregulin-1 isoform, blocks axon ensheathment and myelination, and leads to extensive demyelination when added to mature cocultures. These findings suggest a potential role for the neuregulins and other mitogens as inhibitors of Schwann cell differentiation, and support their role in pathologic demyelination as discussed below.

Schwann Cells Exit the Cell Cycle in the Presence of Saturating Amounts of Neuregulins

The neuregulins are known to be major components of the contact-dependent neurite mitogen that promotes Schwann cell proliferation during development (Morrissey et al., 1995). In contrast, the signals that cause Schwann

cells to exit the cell cycle have not been identified. Potentially, limiting amounts of neuregulins, and/or downregulation of their receptors, could be responsible. To investigate these possibilities, we added GGF to cocultures either during the period of rapid Schwann cell proliferation or in older, myelinating cultures in which most Schwann cells had stopped dividing. Addition of GGF did not further increase the mitotic index of Schwann cells rapidly proliferating in association with neurons (data not shown); presumably, they were already maximally stimulated by neuregulins on the neurites (Morrissey et al., 1995). In mature cocultures that had myelinated for 3 wk, GGF modestly increased the labeling index (Table I) and the resultant density of Schwann cells (Fig. 6 D). Even in the presence of GGF, however, the great majority of the Schwann cells associated with neurites in mature cultures did not reenter the cell cycle despite robust erbB phosphorylation (Fig. 7). These results suggest that downregulation of neuregulin-erbB signaling between axons and Schwann cells (discussed further below) only modestly contributes to Schwann cell cycle arrest and that other signals, such as density-dependent interactions between Schwann cells, play a more significant role (Casella et al., 2000).

Role of the Neuregulins in Regulating Myelination

In this report, we have demonstrated that physiologic concentrations of GGF and FGF-2 strikingly inhibit myelination in Schwann cell-neuron cocultures. These results are consistent with the effects of these mitogens on myelin protein expression by forskolin-treated Schwann cells (Morgan et al., 1994; Cheng and Mudge, 1996) and extend previous studies in which TGF-β was found to potently block myelination in cocultures (Einheber et al., 1995; Guénard et al., 1995). Together, they suggest that mitogen activation can maintain Schwann cells in a pre- or nonmyelinating state and raise the possibility that such mitogens may correspond to the inhibitory signals that promote the nonmyelinating phenotype of Schwann cells in unmyelinated nerves (Lee et al., 1997). However, as Schwann cells in the GGF-treated cultures do not ensheathe or segregate axons appropriately (Fig. 2), the neuregulins may not fully promote the ensheathing phenotype in vivo.

These studies contrast with a recent report (Garratt et al., 2000b) suggesting that erbB2 promotes myelination. In particular, mice with a prenatal conditional knockout of erbB2 exhibited significant hypomyelination, including thinly myelinated and amyelinated large caliber axons. Similarly, activation of PI 3-kinase in Schwann cells, which is largely driven by neuregulin-1 on the axon surface (Maurel, P., and J. Salzer, unpublished studies), is required for the early events of myelination but not for myelin maintenance (Maurel and Salzer, 2000). Both studies do not distinguish between a role for erbB2 or PI 3-kinase in establishing initial axon-Schwann cell interactions that are a prelude to myelination or a direct role during myelination itself; thus, in the case of the conditional erbB2 knockout, it may have been ablated as early as E16. In addition, erbB2 is present in the abaxonal membrane of myelinating Schwann cells (Carroll et al., 1997), a distribution we have also observed in the cocultures (Zanazzi, G., and J. Salzer, unpublished observations). In this location, erbB2 would be unlikely to mediate signals from axonal neuregulins, but rather would be more likely to interact with ligands present in the extracellular matrix, including, potentially, other members of the EGF superfamily. ErbB2 may also regulate the activity of integrins (Gambaletta et al., 2000), which are similarly concentrated in the abaxonal membrane (Einheber et al., 1993) and are required for myelination (Fernandez-Valle et al., 1994). In the future, studies to examine directly the requirement for NRG-1 at different stages of myelination will be of interest.

Taken together, these studies suggest that neuregulin signaling, in particular activation of PI 3-kinase, is required to generate Schwann cells and establish axon-Schwann cell relationships required for the early morphogenetic events of myelination, whereas, once a full complement of Schwann cells is generated and organized into a 1:1 relationship with axons, persistent neuregulin signaling inhibits myelination. This provides a mechanism for preventing Schwann cells whose abaxonal surface is in contact with an unensheathed nerve fiber from forming myelin, ensuring that myelination would only commence once all axons are ensheathed. These results are also consistent with evidence that neuregulin signaling is substantially downregulated during myelination and in the adult. Thus, the levels of some neuregulin isoforms (Chen et al., 1994; Shinoda et al., 1997), erbB2 (Cohen et al., 1992) and erbB3 (Grinspan et al., 1996), are all downregulated during postnatal development, as is CD44, which enhances neuregulin signaling (Sherman et al., 2000). In agreement, erbB2 is abundantly phosphorylated in the perinatal sciatic nerve (Grinspan et al., 1996), but is minimally phosphorylated in the adult (Grinspan et al., 1996; Kwon et al., 1997). Decreased expression of the neuregulins, as well as the substantial downregulation of their receptors, together with the redistribution of these receptors to the abaxonal surface, would be expected to substantially reduce neuregulin signaling by axons, thereby allowing myelination to proceed.

GGF Activates Mitogen Signaling Pathways and Results in Dedifferentiation

GGF treatment of myelinating cocultures results in rapid erbB phosphorylation and activation of the MAP kinase and PI 3-kinase intracellular signaling pathways. These initial events are likely to play a key role in subsequent demyelination as evidenced by the striking demyelination observed with even a brief 30-min pulse of GGF; in addition, the dose-dependent increase in the p185 phosphorylation (Fig. 7) correlated well with the dose-dependent demyelination (Fig. 3 C). Tyrosine phosphorylation of p185 was rapidly followed by phosphorylation and dephosphorylation of a 120-kD protein. Interestingly, GGF activated p120 phosphorylation only in myelinated cocultures, suggesting that this protein is specifically expressed in myelinating Schwann cells and may have a role in GGF-induced demyelination. The identity of this protein, whether it is specifically phosphorylated by neuregulins but not other mitogens and whether it is phosphorylated during demyelination in vivo are important questions for future investigation.

In contrast to the rapid activation of these signaling pathways, GGF-induced demyelination was apparent after a significant lag of 24–36 h, however, presumably reflecting downstream effects including changes in transcription factor levels and activities. Neuregulins regulate the activity of several transcription factors (Hagedorn et al., 2000; Talukder et al., 2000) and result in sustained CREB phos-

phorylation in Schwann cells (Tabernero et al., 1998); this latter effect may relate to the ability of GGF to induce SCIP expression in myelinating Schwann cells. This induction indicates that GGF caused the myelinating Schwann cells to dedifferentiate to the promyelinating stage, which is characterized by high SCIP expression (Zorick et al., 1996; Arroyo et al., 1998). Interestingly, GGF blocks the differentiation of oligodendrocyte progenitors and causes oligodendrocytes to dedifferentiate to the pro-oligodendrocyte stage (Canoll et al., 1996, 1999). Thus the effects of the neuregulins on oligodendrocytes and Schwann cells (i.e., promoting the generation of myelinating glial cells and inhibiting their differentiation) is well conserved between these two cell types.

An important question is whether altered expression of transcription factors is mechanistically related to the demyelination observed with GGF treatment. SCIP inhibits the transcription of P0 (Monuki et al., 1990), and its increased expression might therefore affect myelin production and stability; it also increases transiently during Wallerian degeneration (Zorick et al., 1996). However, as reduction of myelin protein expression alone is insufficient to induce Wallerian degeneration (Toews et al., 1997), the neuregulins may actively promote myelin sheath degeneration by other mechanisms.

Relationship of Proliferation to Demyelination

In addition to demyelination, GGF induced Schwann cell proliferation in the myelinating cultures. Demyelination itself is not dependent on earlier Schwann cell proliferation, as many cells that did not proliferate during the treatment period exhibited substantial myelin degeneration (Fig. 6 C). Indeed the majority of demyelination occurred in BrdU⁻ cells. Proliferation of previously myelinating Schwann cells was apparent by 36 h and increased further at 48 h of GGF treatment; at this stage, demyelination was quite advanced, suggesting that proliferation may occur as a later event. In agreement, Schwann cells that proliferated were at advanced stages of myelin degeneration (Table I). These results suggest that either breakdown products of myelin are themselves mitogenic (Salzer and Bunge, 1980; Bigbee et al., 1987) or, conversely, that myelin debris must be substantially cleared and Schwann cells must dedifferentiate before GGF is able to induce Schwann cells to reenter the cell cycle (Fernandez-Valle et al., 1995). We now favor the latter possibility as the occasional Schwann cell that spontaneously demyelinates in control cultures (i.e., in the absence of GGF) almost never proliferates, even when demyelination is advanced (Zanazzi, G., and J. Salzer, unpublished observations). These results also suggest that, as myelin debris is cleared and possibly erbB expression is upregulated, GGF is able to drive Schwann cell proliferation more efficiently.

Implications for Peripheral Neuropathies and Wallerian Degeneration

Demyelination and Schwann cell proliferation are also hallmarks of Wallerian degeneration and hypertrophic demyelinating neuropathies (reviewed in Asbury and Johnson, 1978; Scherer and Salzer, 1996). The signals that initiate demyelination in Wallerian degeneration and in various neuropathies are poorly understood. In the case of Wallerian degeneration, two distinct hypotheses have been proposed: disruption of the nerve fiber results in loss of axonal signals required to maintain the myelin sheaths or, alternatively, positive signals released as a consequence of axotomy initiate this process (Scherer and Salzer, 1996). In support of the latter possibility, diffusible mitogens appear to be released within the endoneurium that stimulate proliferation of a variety of cell types, including ensheathing Schwann cells associated with axons (Griffin et al., 1987). In this study, we have demonstrated that increased neuregulin activity, even in the presence of intact axons, is sufficient to induce important aspects of Wallerian degeneration, providing an experimental counterpart to these in vivo findings.

These results are also consistent with earlier studies that suggested a potential role for the neuregulins during Wallerian degeneration. Neuregulin and erbB expression by Schwann cells increases substantially during Wallerian degeneration (Cohen et al., 1992; Carroll et al., 1997) and demyelination (Hall et al., 1997), suggesting a potential autocrine signaling mechanism. Importantly, erbB2 phosphorylation also increases in the distal stump after nerve transection (Kwon et al., 1997). However, while the increases in neuregulin and erbB expression and erbB2 phosphorylation occur during the period of Schwann cell proliferation, they peak later and persist after this proliferation has declined (Carroll et al., 1997; Kwon et al., 1997). Thus, while neuregulin receptor activation is likely to potentiate Schwann cell proliferation, dedifferentiation, and demyelination in the distal stump, the precise role of this activation, particularly during later stages of Wallerian degeneration, is unclear. Other possible roles include enhancing nerve fiber regeneration by promoting Schwann cell migration into the gap between the proximal and distal stumps (Mahanthappa et al., 1996) and by increasing expression of neurotrophins available to regenerating axons (Verdi et al., 1996). Results from this study also raise the possibility that neuregulins promote the clearance of myelin debris.

In summary, these results suggest that the neuregulins and erbBs play an important role not only in the generation of Schwann cells during development but also in later events of peripheral nerve development and in the adult. Downregulation of neuregulin signaling may be required for myelination to proceed, whereas persistent activity could bias the Schwann cells to the nonmyelinating phenotype. Our results also suggest that activation of mitogen signaling pathways by neuregulins, and possibly other mitogens, in pathologic settings may contribute to dymyelination and the events of peripheral nerve degeneration. Future studies focusing on the role of mitogen signaling pathways during demyelination and the role of the neuregulins in Wallerian degeneration and hypertrophic peripheral neuropathies should further clarify their precise role in these processes.

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