Axonal Neuregulin 1 Type III Activates NF-κB in Schwann Cells during Myelin Formation

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The formation of myelin requires a series of complex signaling events initiated by the axon to surrounding glial cells, which ultimately respond by tightly wrapping the axon with layers of specialized plasma membrane thereby allowing for saltatory conduction. Activation of the transcription factor NF-κB in Schwann cells has been suggested to be critical for these cells to differentiate into a myelinating phenotype; however, the mechanisms by which it is activated have yet to be elucidated. Here, we demonstrate that axonal membranes are sufficient to promote NF-κB activation in cultured Schwann cells and identify neuregulin 1 (NRG1), specifically the membrane-bound type III isoform, as the signal responsible for activating this transcription factor. Surprisingly, neither membrane-bound type I nor the soluble NRG1 EGF domain could activate NF-κB, indicating that type III induces a qualitatively unique signal. The transcriptional activity of NF-κB was significantly enhanced by treatment with forskolin, indicating these two signals converge for maximal activation. Both ErbB2 and -3 receptors were required for transducing the NRG1 signal, because gene deletion of ErbB3 in Schwann cells or treatment with the ErbB2 selective inhibitor, PKI-166, prevented the stimulation of NF-κB by axonal membranes. Finally, PKI-166 blocked the activation of the transcription factor in myelinating neuron/Schwann cell co-cultures and in vivo, in developing sciatic nerves. Taken together, these data establish NRG1 type III as the activator of NF-κB during myelin formation.

Reciprocal communication between axons and Schwann cells in the peripheral nervous system is essential for axonal ensheathment, myelin formation, and axonal maintenance and repair. However, the complex signaling events that regulate Schwann cell maturation and differentiation have yet to be fully understood.

One of the key signals implicated in Schwann cell reprogramming into a myelinating phenotype is the activation of the transcription factor NF-κB (1). NF-κB has been best characterized for its role in the immune system and regulating cell survival (2, 3); however, recent data have linked this transcription factor to the development and differentiation of cells in the nervous system (4). NF-κB is active in the sciatic nerve during peripheral myelin formation and in myelinating dorsal root ganglia (DRG)2/Schwann cell co-cultures. Furthermore, myelination in the co-cultures was prevented by blocking the transcription factor or by genetic deletion of the p65 subunit of NF-κB (1). These data indicate that NF-κB activation in Schwann cells is a critical event for their differentiation into a myelinating phenotype; however, the mechanism by which axons stimulate the transcription factor remains to be elucidated.

Phosphorylation of the p65 subunit of NF-κB by cAMP-dependent protein kinase A (PKA) was recently reported to enhance its transcriptional activity in Schwann cells, and preventing this phosphorylation inhibited myelin formation (5). An axonal-mediated increase in cAMP has long been suggested as an inducer of Schwann cell differentiation (6–8); however, stimulation of PKA alone was not sufficient to induce DNA binding by NF-κB, suggesting that an unknown axonal signal first promotes NF-κB nuclear translocation and DNA binding, then PKA phosphorylation increases its activity.

Among the various axonally derived signals, the growth factor neuregulin 1 (NRG1) has been implicated in regulating nearly all aspects of Schwann cell development, including proliferation, survival, migration, differentiation, and de-differentiation (9). NRG1 has at least 15 isoforms that can be grouped into three types (10). Type I and II isoforms are produced as transmembrane proteins, but the N terminus is typically shed to produce a soluble ligand. In contrast, type III NRG1 is initially produced with two transmembrane regions, but is cleaved to produce a single membrane-pass protein with an intracellular N terminus. All forms of NRG1 contain an EGF-like domain, which is sufficient to activate the ErbB family of tyrosine kinase receptors (10).

Schwann cells express ErbB2 and -3 (11, 12), which are activated by all forms of NRG1. Nevertheless, isoform-specific effects have been reported; for example, soluble type II NRG1 (often referred to as GGF) stimulates Schwann cell proliferation (13) but does not promote Schwann cell differentiation (14) and can even elicit dedifferentiation of myelinating Schwann cells (15). In contrast, membrane-bound type III

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1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

2 The abbreviations used are: DRG, dorsal root ganglia; PKA, protein kinase A; NRG1, growth factor neuregulin 1; EGF, epidermal growth factor; HA, hemagglutinin; BrdUrd, bromodeoxyuridine; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, ERK/MAPK kinase; GGF, soluble type II NRG1; PI3K, phosphatidylinositol 3-kinase.

61614 JOURNAL OF BIOLOGICAL CHEMISTRY
NRG1 elicits Schwann cell differentiation and promotes myelin formation (14, 16). How such isoform-selective effects are mediated by the ErbB2/3 receptors remains largely unknown.

We report here that membrane-bound type III NRG1 is the axonal signal responsible for initiating NF-κB activation in Schwann cells during myelination. We show both in vitro and in vivo that stimulation of NF-κB by axons requires ErbB2/3 receptor activity. These results demonstrate that the activation of a promyelinating transcription factor is specifically regulated by the type III NRG isoform.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Antibodies raised against ERK (p42 and p44), phospho-ERK, Akt, phospho-Akt, phospho-p65, and ErbB3 were obtained from Cell Signaling Technology. Antibodies recognizing Erk2, ErbB2, phospho-ErbB2, and p65 were purchased from Santa Cruz Biotechnology. Antibodies recognizing ErbB2, ErbB3, phospho-ErbB2, and p65 were obtained from Cell Signaling Technology. Antibodies recognizing Erk2, ErbB2, phospho-ErbB2, and p65 were obtained from Cell Signaling Technology. Antibodies recognizing Erk2, ErbB2, phospho-ErbB2, and p65 were obtained from Cell Signaling Technology.

Cell Culture—COS7 cells were maintained in DMEM with 10% fetal bovine serum (FBS, Sigma) and penicillin/streptomycin (Invitrogen). All experiments using animals were approved by the Animal Care and Use Committee at Vanderbilt University. Rat Schwann cells were isolated from sciatic nerves of 4- to 5-day-old Sprague-Dawley rats and purified as previously described in Yoon et al. (5). Rat Schwann cells were grown with 2 μM forskolin in DMEM with 10% FBS (Sigma). Mouse Schwann cells were isolated from sciatic nerves of CD1 postnatal day 4 to 5 mice (17), plated on poly-l-lysine-coated dishes, and maintained in DMEM with 10% FBS (Sigma) initially supplemented with 25 ng/ml NRG1 (recombinant EGF domain). Mouse Schwann cells were washed in PBS and maintained in low serum media (1–2% FBS) without NRG1 prior to stimulation.

Mouse DRG were isolated at embryonic day 14 and DRG explants were plated on poly-l-ornithine and laminin-coated dishes in Neurobasal media (Invitrogen) supplemented with B-27 (Invitrogen), l-glutamine, and 50 ng/ml NGF (Harlan Bio-products, Madison, WI). The DRG cultures were pulsed with 5–10 μM cytosine arabinoside for two 24-h treatments to remove non-neuronal cells.

Myelinating DRG/Schwann cell co-cultures were established using DRGs isolated from E15 rats as described (1, 5) and plated in Ultraculture media (BioWhittaker) supplemented with 10% FBS (HyClone), 2 mM l-glutamine (Invitrogen), and 50 ng/ml NGF (Harlan) at a density of 80,000 cells/2.2 cm² collagen-coated coverslip. Myelination was induced 5 days later by adding 50 μg/ml ascorbic acid in growth media. Growth media and ascorbic acid were replaced every 2 days.

Membrane Purification—Sensory neuron membranes were isolated from DRG neurons, initially plated as explants but treated with cystosine arabinoside to remove all non-neuronal cells as described before (13, 18) with modifications. The neurons were rinsed in PBS, and cell bodies were excised using a scalpel under a dissecting microscope. Following removal of cell bodies, neurites were lifted from the dishes using forceps. Neurites were homogenized in PBS with a 1 ml Dounce homogenizer and centrifuged at 80 × g at 4 °C to remove debris. The supernatants were then diluted in PBS and centrifuged at 35,000 × g for 1 h at 4 °C to pellet the membranes. The pellet was resuspended in PBS and immediately added to cells. Aliquots of purified membrane homogenate were used for protein assays and Western blotting.

COS7 cells were transfected with either NRG1 type I or NRG 1 type III constructs (19) using Lipofectamine (Invitrogen) according to the manufacturer’s directions. Transfected and untransfected COS7 cells were rinsed in PBS, and then scraped into homogenization buffer (20 mM Hepes, pH 8.0, 1.5 mM MgCl₂, and 1 mM EDTA). Cells were incubated on ice for 10 min and then homogenized in a Dounce homogenizer. Homogenates were centrifuged at 1,000 × g for 10 min at 4 °C, and the supernatants were collected and centrifuged at 35,000 × g for 1 h at 4 °C. The membrane pellets were resuspended in PBS and immediately added to cells. Aliquots of purified membrane were used for protein assays and Western blotting.

Luciferase Assay—Isolated mouse Schwann cells were transfected with both a NF-κB luciferase reporter construct, kindly provided by M. Chao (New York University, New York, NY), and a Renilla construct (Promega) using Effectene (Qiagen) according to the manufacturer’s instructions. 24 h post transfection, the cells were stimulated for an additional 24 h with isolated membranes then lysed, and luciferase activity was analyzed with a Dual luciferase assay kit (Promega), according to the manufacturer’s directions, and an OPTOCOMP 1 Version 1.10 luminometer from CMGM Instruments (Hamden, CT).

Immunoprecipitation and Western Blotting—Phospho-p65 immunoprecipitation from rat Schwann cells was performed as previously described (5). For immunoprecipitation experiments, confluent rat Schwann cells were cultured in DMEM plus 10% FBS for 2 days then in a 1:1 mixture of DMEM and Ham’s F-12 with N2 supplement (Invitrogen), for another 2 days. Cells were then treated for 1 h with forskolin (20 μM), dibutyryl-cAMP (500 μM, Biomol Research Laboratories, Plymouth Meeting, PA), or membrane fragments and lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM MgCl₂, 1 mM EGTA, 1 mM Na₃VO₄, 20 mM β-glycerol phosphate, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche Applied Science)). The lysates were dissociated by sonication and cleared by centrifugation at 13,000 × g for 10 min at 4 °C, and protein concentrations were determined by Bradford assay (Bio-Rad). Cell lysates (0.5–1 mg of protein) were incubated with 2 μg of p65 antibody (Rockland Immunochemicals, Gilbertsville, PA) overnight at 4 °C with rotation and then immunoprecipitated with protein G-agarose beads (Zymed Laboratories Inc.). The
NRG1 Type III Activates NF-κB in Schwann Cells

immunoprecipitates were separated by SDS-PAGE and Western blotted with antibodies to p65 (1:2,000, Rockland Immunocyticals) or phospho-Ser-276 p65 (Cell Signaling Technology, Beverly, MA).

In some cases lysates of mouse Schwann cells were subjected to SDS-PAGE and Western blotting without immunoprecipitation. The membranes were blocked in Tris-buffered saline, pH 7.4, with 0.1% Tween (TBST) containing 3% bovine serum albumin, treated with primary antibody overnight. Secondary antibodies conjugated to horseradish peroxidase were added. Alternatively, secondary antibodies conjugated to fluorescent IRdyes (LI-COR Biosciences) were used to detect proteins in conjunction with the Odyssey Infrared Imaging System (LI-COR Biosciences).

Electrophoretic Mobility Shift Assay—NF-κB was analyzed by gel-shift assay with a 32P-labeled, double-stranded NF-κB consensus oligonucleotide (Promega) as previously described (20). An OCT1 oligonucleotide (Promega) was used as a control. Antibodies to p65/RelA (Rockland Immunocyticals and Santa Cruz Biotechnology, Santa Cruz, CA) were used for supershift analysis.

ErbB3 Deletion in Schwann Cells—Schwann cells were isolated from postnatal day 4–5 mice homozygous for a floxed ErbB3 allele previously described (21). The cells were infected with an adenovirus expressing green fluorescent protein or Cre recombinase (22). Viral dilutions were optimized for each virus on the Schwann cells to obtain >95% infectivity, using green fluorescent protein fluorescence or immunostaining with anti-Cre conjugated to biotin (1:100, Covance), followed by incubation with streptavidin-Cy3. 48 h after infection, the cells were lysed and subjected to electrophoretic mobility shift assay analysis and Western blotting as indicated.

BrdUrd Assay—Mouse Schwann cells were plated on poly-L-lysine-coated slides in DMEM with 2.5% FBS and penicillin/streptomycin (Invitrogen). The cells were incubated in the presence or absence of 50 μg/ml SN50 (Biomol) and treated with membranes from neuregulin-expressing or control COS7 cells twice, each time for 24 h. Following the treatments, 20 μM BrdUrd (Sigma) was added for an additional 4 h. The cells were then fixed in 3:1 methanol to glacial acetic acid at −20 °C for 20 min followed by treatment with 2 N HCl for 20 min at 37 °C and by 0.1 M boric acid for 2 min at room temperature. The slides were blocked in 8% bovine serum albumin in PBS with 0.1% Triton X-100 for 1 h, incubated overnight at 4 °C with anti-BrdUrd (DAKO) and anti-p75 neurotrophin receptor (to confirm the identity of the Schwann cells), then visualized using rhodamine-conjugated anti-mouse (Pierce) and anti-rabbit conjugated to Alexa Fluor 488 (Molecular probes) and a Zeiss Axioscope fluorescence microscope.

Sciatic Nerve Injections—Postnatal day 1 (P1) rat pups were intraneurally injected in their right sciatic nerve with 1.4 mg/kg PKI-166 dissolved in DMSO in a total injection volume of 2.5 μl. The left sciatic nerve was injected with saline or with vehicle alone. One hour post injection, the sciatic nerves were removed, lysed in lysis buffer, dissociated by sonication, and centrifuged at 13,000 × g for 10 min at 4 °C. The protein concentration of the supernatant was determined by Bradford and analyzed by Western blot for phosphorylated ERK to confirm PKI-166 incorporation into the sciatic nerve and inhibition of ErbB2. Samples were then tested for NF-κB activation.

RESULTS

Axonal Membranes Activate NF-κB in Cultured Schwann Cells—Schwann cells in the perinatal sciatic nerve as well as in culture with DRG neurons in vitro display activated NF-κB, and this signal was necessary for myelin formation (1, 5). Because myelination requires axonally derived signals, we hypothesized that axonal contact is responsible for activating NF-κB. To test this hypothesis, isolated mouse Schwann cells were treated with purified DRG axonal membrane fragments, and the activity of NF-κB was measured. Exposure to the axonal membranes for 30, 60, and 90 min elevated NF-κB binding to DNA (Fig. 1A), suggesting that an axonal membrane-associated ligand is sufficient to induce DNA binding by NF-κB in Schwann cells. Binding of an unrelated transcription factor, OCT1, was unaffected by axon stimulation (Fig. 1C).

Previous studies from our laboratory demonstrated that phosphorylation of the p65 subunit of NF-κB on serine 276 by PKA enhanced its transcriptional activity but did not increase DNA binding (5). Therefore, we investigated whether isolated axonal membranes were sufficient to fully activate NF-κB. Following treatment of Schwann cells with axonal membranes, we did not observe any increase in the phosphorylation of p65 on Ser-276 as compared with controls (Fig. 1D). In contrast, Ser-276 was phosphorylated in response to dibutyryl-cAMP, as previously reported (5). In accord with phosphorylation being necessary for maximal transcriptional activity, axon membranes alone only modestly increased NF-κB transcription, based on a luciferase reporter assay (Fig. 1E). However, combining the axon fragments with the cAMP activator forskolin resulted in a synergistic activation. Forskolin treatment alone was unable to stimulate NF-κB binding to DNA (Ref. 5 and data not shown). Additionally, the PKA inhibitor, H89, had no effect on either the axonal stimulation of NF-κB-DNA binding (Fig. 1G) or transcriptional activity (Fig. 1F). These results indicate that an axon membrane-associated factor can stimulate NF-κB binding to DNA, but an additional signal is required for maximal transcriptional activity.

NRG1 Type III, but Not Other NRG1 Isoforms, Stimulates NF-κB—NRG1 type III is expressed on the surface of axons and known to be essential for Schwann cell development and myelination (14, 16, 18, 23–26). Furthermore, the neuregulin receptor ErbB2, activated in breast cancer cells, has been shown to stimulate NF-κB (27). Therefore, we considered type III NRG1 a likely candidate for the axonal signal activating this transcription factor. To determine if this membrane-bound ligand could stimulate NF-κB in Schwann cells, we transfected COS7 cells with NRG1 type III and isolated membrane fractions. In addition, NRG1 type I was transfected into the COS cells to compare the effects of the different types of NRG1. After confirming the expression of both type I and III in the isolated membranes (Fig. 2B), we treated Schwann cells with membrane fragments expressing NRG1 type I or type III or from untransfected cells, DRG axon membranes served as a positive control. Interestingly, NF-κB activity was up-regulated only in response
NRG1 Type III Activates NF-κB in Schwann Cells

Axonal membranes activate NF-κB in cultured Schwann cells. A, axonal membrane fragments were isolated from DRG neurons and added to cultured Schwann cell for 30, 60, or 90 min, and NF-κB activity was assessed by gel shift. To confirm the identity of the shifted band, excess unlabeled probe (cold) or an antibody to p65 (α-p65 ab) were added. p65 antibody shifted the band and reduced binding to the probe (n = 4). B, nonspecific rabbit IgG was unable to shift the p65-DNA complex; and C, there was no change in the binding of OCT1, an unrelated transcription factor.

D, cultured rat Schwann cells were incubated in defined media for 48 h (5), then stimulated with axons for 24 h. NF-κB activity was analyzed by luciferase assay (n = 4).

E, mouse Schwann cells were transfected with an NF-κB reporter construct, as well as an expression construct for Renilla, and stimulated with 50 ng/ml GGF, 20 μM forskolin, 50 ng/ml tumor necrosis factor-α, axonal membranes, or axonal membranes with forskolin for 24 h. NF-κB activity was analyzed by luciferase assay (n = 4). F, mouse Schwann cells were transfected with an NF-κB reporter construct, as well as an expression construct for Renilla, and treated with or without 10 μM H89 and then stimulated with axons for 24 h. NF-κB activity was analyzed by luciferase assay. G, Schwann cells were treated with or without 10 μM H89 and then stimulated with axons for 1 h. NF-κB-DNA binding was analyzed by electrophoretic mobility shift assay.

FIGURE 1. Axonal membranes activate NF-κB in cultured Schwann cells. A, axonal membrane fragments were isolated from DRG neurons and added to cultured Schwann cell for 30, 60, or 90 min, and NF-κB activity was assessed by gel shift. To confirm the identity of the shifted band, excess unlabeled probe (cold) or an antibody to p65 (α-p65 ab) were added. p65 antibody shifted the band and reduced binding to the probe (n = 4). B, nonspecific rabbit IgG was unable to shift the p65-DNA complex; and C, there was no change in the binding of OCT1, an unrelated transcription factor. D, cultured rat Schwann cells were incubated in defined media for 48 h (5), then stimulated with axonal membranes or 500 μM dibutyryl-cAMP for 1 h. Cells were lysed, and immunoprecipitations were performed with an anti-p65 antibody. Subsequent Western blots were probed with an antibody specific for the phospho-Ser-276 residue on p65. Results are shown as the amount of phosphorylation over control levels (n = 4).

Activating ERK and PI3K Is Required for NF-κB Stimulation—Axonal membranes and NRG1 isoforms can activate both the PI3K and MAPK signaling pathways (18). Both these pathways have been demonstrated to activate NF-κB in multiple cell types (30–33). To determine if either of these pathways was required for the activation of NF-κB in Schwann cells by axonal fragments, we utilized the chemical inhibitors LY294002 and PD98059, which inhibit the PI3K and MAPK pathways, respectively (Fig. 3, A and B). Pretreatment with either LY294002 or PD98059 was sufficient to block the axon membrane-induced activation of NF-κB (Fig. 3C). Similar effects were seen with wortmannin, another inhibitor of PI3K.
NRG1 Type III Activates NF-κB in Schwann Cells

FIGURE 2. NRG1 type III, but not other NRG1 isoforms, stimulates NF-κB activity. A, primary mouse Schwann cells were treated for 1 h with either axonal membranes or COS7 cellular membranes that were either untransfected (UnT) or expressing NRG1 type I or NRG1 type III. NF-κB activation was detected by gel shift analysis. The identity of the band was confirmed by inhibiting binding with an antibody to p65 and by adding excess unlabeled probe (cold). Quantification of n = 3 (right panel). B, COS7 cells were transfected with constructs expressing either NRG1 type I or type III. Membrane fractions were isolated and analyzed by Western blot for expression of the NRG1 isoforms. NRG1 type I was detected by anti-HA, whereas NRG1 type III was detected with an antibody specific for this isoform. C, primary mouse Schwann cells were treated for 1 h with either axonal membranes or 100 ng/ml of soluble type II NRG1 (GGF) and analyzed for NF-κB activation by gel shift (n = 4). D, Schwann cells were treated with 50 ng/ml GGF, or membranes expressing NRG1 type I or type III, or axonal membranes for 1 h. ERK activation was analyzed by Western blotting with antibodies to phospho-ERK and total ERK2. Quantification of n = 3–5 (right panel), all treatments significantly elevated ERK phosphorylation over control (p < 0.05). E, Schwann cells were treated with 50 ng/ml GGF or NRG1 type I expressing membranes for the indicated times. Cell lysates were probed with antibodies to phospho-Akt and total Akt. Right panel, quantification of n = 4–5; all treatments significantly elevated Akt phosphorylation over control (p < 0.05).

FIGURE 3. Activation of ERK and PI3K are required for NF-κB stimulation. A, primary mouse Schwann cells were pretreated with or without 20 μM LY294002 for 30 min and then stimulated with axonal membranes for 1 h. Activation of the PI3K pathway was analyzed by Western blots probed for phospho-Akt and total Akt. B, mouse Schwann cells were pretreated with or without 50 μM PD98059 for 30 min and then stimulated with axonal membranes for 1 h. Activation of the MAPK pathway was analyzed by Western blots probed for phospho-ERK and total ERK. C, Schwann cells were pretreated with or without 20 μM LY294002 or 50 μM PD98059 and stimulated with axonal membranes for 1 h. NF-κB activity was determined by gel shift analysis. (data not shown). These results suggest that both the PI3K and ERK pathways contribute to the activation of the transcription factor.

NF-κB Stimulation by Axonal Membranes Requires ErbB2 and ErbB3 Activity—Axons express type III NRG1 (14, 25, 26), and our results demonstrate that this specific isoform, when expressed in COS7 cells, can activate NF-κB (Fig. 2A). These data suggest that this growth factor is the axonal signal responsible for stimulating the transcription factor in Schwann cells. To further explore this possibility, we assessed the requirement for the neuregulin receptors. ErbB2/3 heterodimers serve as the functional NRG1 receptor on Schwann cells (11, 12), with ErbB2 providing the catalytically active kinase domain and ErbB3 the ligand binding, extracellular domain. To determine whether ErbB2 signaling is necessary for axonal activation of NF-κB, Schwann cells where pretreated with the ErbB2 kinase inhibitor PKI-166 (for review of PKI-166 properties see Traxler et al. (34–37)), and then stimulated with axonal membranes. Inhibition of receptor signaling was confirmed by assessing ERK and Akt activation (Fig. 4, A and B). Importantly, the PKI-166-treated cells exhibited reduced NF-κB activity following axonal stimulation (Fig. 4, C and D), indicating that axons signal through ErbB2 to activate this transcription factor in Schwann cells.

We next queried whether ErbB3 also participates in the axonal signal to NF-κB. Schwann cells were isolated from transgenic mice harboring a conditional allele for ErbB3 (21) and the cells infected with an adenovirus expressing Cre recombinase or green fluorescent protein, as a control. After 48 h, ErbB3 protein expression and axonal membrane-mediated activation of ERK and Akt were attenuated (Fig. 5A). Importantly, the activation of NF-κB in ErbB3-deficient Schwann cells by axonal membranes was substantially reduced (Fig. 5B). Together,
these results suggest that axonal NRG1 type III stimulation of ErbB2/3 receptors on Schwann cells results in the activation of NF-κB.

NF-κB Activation Is Dispensable for Schwann Cell Proliferation—Although neuregulin can induce Schwann cell differentiation (14, 25, 26), this growth factor is also known to promote Schwann cell proliferation (18). In addition, activation of NF-κB can stimulate cell proliferation in some contexts (38). Therefore, to determine if NF-κB activity contributes to NRG1-mediated cell proliferation, we incubated Schwann cells with SN50, a membrane-permeable inhibitor of NF-κB (39) (Fig. 6A) that has previously been demonstrated to have no effect on p65 expression in unstimulated cells (40). We then treated the cells with GGF, NRG1 type III-expressing membranes, or untransfected membranes. After 48 h of treatment, cell proliferation was then measured by a 4-h BrdUrd pulse (Fig. 6B). In agreement with the aforementioned studies, Schwann cells treated with GGF or NRG1 type III-expressing membranes displayed increased proliferation over untreated cells; however, inhibition of NF-κB had no significant effect, demonstrating that NF-κB activity does not contribute to Schwann cell proliferation stimulated by neuregulin. Similar results were obtained when NF-κB was inhibited by infecting Schwann cells with adenoviruses expressing a mutant form of IκB (supplemental Fig. 1). These data are in agreement with previous findings from our laboratory that demonstrate overexpression of mutant IκB in Schwann cells has no effect on cellular survival or proliferation but prevents myelination (1).

ErbB2 Mediates the Activation of NF-κB during Myelin Formation—The preceding results demonstrate that membrane-bound NRG1 type III is able to stimulate NF-κB in purified Schwann cells; therefore, we sought to determine whether neuregulin signaling was responsible for the activation of this transcription factor during peripheral myelin formation. We
first utilized an in vitro myelination assay where Schwann cells were co-cultured with DRG neurons, and the formation of myelin was induced by the addition of ascorbic acid (1, 41). In agreement with previous findings (1), we detected robust NF-κB activation in myelinating co-cultures following 6 days of ascorbic acid treatment (Fig. 7). However, in co-cultures treated for 2 h with the ErbB2 inhibitor, PKI-166, NF-κB activity was markedly reduced in PKI-166-injected nerves (Fig. 7C). These data indicate that NRG1 signaling through ErbB2 receptors is essential for the activation of NF-κB during peripheral myelin formation.

**DISCUSSION**

Previous studies implicated NF-κB as a critical factor in regulating Schwann cell differentiation into a myelinating phenotype (1, 5); however, how this transcription factor is activated remained poorly understood. Here, we identify neuregulin 1 type III as the axonal signal responsible for stimulating NF-κB in Schwann cells, both in vitro and in vivo during peripheral myelin formation. Both soluble and membrane-bound NRG1 isoforms activate the identical receptor complex (ErbB2/3) on Schwann cells, but elicit different biological effects (25, 42). Soluble NRG1 isoforms type I and II promote survival and proliferation (18), whereas the membrane-bound isoform type III stimulates the expression of differentiation markers such as OCT6 (25) and P0 (42). How such differing effects can be elicited from the same receptor complex was not known. Our results demonstrate that type III NRG1 uniquely activates NF-κB, a signal associated with Schwann cell differentiation, thus providing one pathway specific to this isoform.

Although NRG1 type III stimulated DNA binding by NF-κB, it only modestly increased transcription. We found that co-stimulation with cyclic AMP (cAMP) substantially enhanced the transcriptional activation. This finding supports our previous results that elevated cAMP in Schwann cells leads to PKA-dependent phosphorylation of NF-κB on Ser-276 of the p65 subunit, which enhanced its transcriptional activity but did not increase DNA binding (5). Both NF-κB binding to DNA and the phosphorylation of p65 were required for myelination in co-cultures of sensory neurons and Schwann cells. Taken together, these findings delineate two signaling pathways that converge to fully activate NF-κB during myelin formation: First, NRG1 type III induces NF-κB binding to DNA, and then PKA phosphorylates the p65 subunit, thereby enhancing NF-κB transcriptional activity.

Because neurons express NRG1 type III and elevate cAMP levels in Schwann cells, it is difficult to differentiate the role of each pathway in myelination in vivo or in Schwann cell/DRG co-cultures. However, the data presented here in concert with our previous work allow us to hypothesize distinct roles of NRG1 type III and cAMP in the activation of NF-κB. NF-κB binding to DNA was detected only in Schwann cells exposed to NRG1 type III. Elevation of cAMP through forskolin or dibutyryl-cAMP alone was unable to elicit NF-κB DNA binding. Furthermore, inhibition of PKA was unable to reduce axonal membrane-stimulated NF-κB DNA binding or NF-κB activity. Taken together these data indicate that cAMP is unable to stimulate NF-κB binding to DNA and is dispensable for the axon-mediated formation of an NF-κB-DNA complex. However, cAMP elevation allows for the phosphorylation of NF-κB on Ser-276. Axonal membranes alone are unable to elicit this phosphorylation. Previous data from our laboratory indicate that Schwann cells expressing a p65 mutant that cannot be phosphorylated on Ser-276 form less myelin than cells expressing wild-type p65 (5). These data allow us to hypothesize that NF-κB-DNA binding elicited by axonal membranes and NF-κB phosphorylation induced by cAMP are both required for maximal myelin formation.

A similar convergence of NRG1 and cAMP pathways in the regulation of other signaling proteins in Schwann cells has pre-
Recently, the role of the neurotrophin receptor p75 in NF-κB activation and the use of MEK inhibitors to reduce NF-κB-mediated transcription (31, 32). Our data indicate that the inhibition of ERK in Schwann cells also attenuates NF-κB activity.

Our results demonstrate that the type III isoform of NRG1 can activate NF-κB, even though the same receptor complex is activated by other isoforms. The ability of different ligands to elicit alternate downstream signals through the same receptor is not without precedent. Receptor tyrosine kinases like ErbB2 and -3 induce downstream signaling by recruiting adaptor proteins that bind to distinct phosphotyrosine sites. One mechanism of effecting disparate signaling is for individual ligands to effect the phosphorylation of different tyrosine residues. This mechanism for ligand discrimination has been described in ErbB4 signaling, where stimulation with the EGF-like ligands, betacellulin, NRG1, or NRG2, all allow for ErbB4 receptor dimerization and phosphorylation; however, phosphopeptide mapping of these receptors revealed that each ligand stimulated the phosphorylation of a unique subset of tyrosine residues on the dimerized receptor complex (46). Furthermore, NRG1 and NRG2 stimulated ErbB4 binding to the p85 subunit of PI3K, whereas betacellulin and NRG1 allowed for the adaptor protein Grb2 to bind to the receptor complex (46). Similarly, NRG1 but not NRG2 was able to simulate the recruitment of Grb2 to the ErbB2/3 complex (47), and differential activation of ErbB2/3 receptors by different NRG isoforms induced distinct profiles of gene expression (48). These data support our finding that ErbB2/3 receptors can discriminate between two NRG1 ligands in Schwann cells and elicit unique signaling events based on the stimulating ligand.

Previous studies from our laboratory have examined the role of the neurotrophin receptor p75 in NF-κB activation and reported that NGF binding to p75 can stimulate NF-κB (20). p75 has also been demonstrated to play an essential role in myelin formation, and p75 knock-out animals display reduced myelination in the peripheral nervous system (49). Furthermore, treatment with the neurotrophin brain-derived neurotrophic factor enhances myelin formation in Schwann cell/DRG co-cultures through a p75-dependent pathway (49). Despite these documented roles of p75 in both NF-κB activation and myelin formation, we did not observe reduced NF-κB activity in p75-deficient nerves during myelin formation (data not shown), suggesting that p75 does not regulate NF-κB activity required for myelin formation.

One complicating factor in analyzing the differences between soluble and membrane-bound NRG1 isoforms is the presence of the membrane itself. The presence of a protein-rich membrane also allows for the possibility that other membrane-bound factors can modify the NRG1 type III/ErbB signaling pathway. Indeed, NRG1 type III expressed on the surface axons was shown to induce myelination by Schwann cells, but its expression in non-neuronal cells was insufficient to induce differentiation into a myelinating phenotype, indicating that other factors on the axonal membrane are required (26). Correspondingly, we find that COS7 cell expression of NRG1 type III is sufficient to stimulate NF-κB binding to DNA; however, it was unable to stimulate the phosphorylation of p65 required for myelin formation.

Previous studies have demonstrated other factors are able to modulate ErbB signaling. For example, the ectodomain of E-cadherin was shown to induce dimerization and activation of the ErbB2/3 complex in breast cancer cells, leading to increased ERK activity (50). Therefore, we cannot rule out the possibility that additional factors on the cellular membrane may be able to modulate NRG1 type III activation of the receptor complex, possibly altering the intensity or duration of the signaling pathways elicited by the receptor and allowing for NF-κB activation. Finally, it is possible that differential signaling between membrane-bound and soluble NRG1 is an effect of the physical localization of the ligand-receptor complex. NRGs tethered to the axonal membrane are unlikely to be as readily internalized as soluble isoforms. Persistent receptor signaling from the cell surface may result in differential effector activation, leading to alternative biological outcomes. It will be interesting to address ErbB2/3 receptor internalization and signal attenuation following stimulation with soluble and membrane-bound NRG1 isoforms in future studies.

NRG1 isoforms regulate Schwann cell development at virtually every stage of development, from neural crest precursors to myelin forming cells, influencing cell survival, proliferation, migration, differentiation, axonal wrapping, and even dedifferentiation (9). Therefore, delineating differential signaling events elicited by different NRG1 isoforms will allow us to char-
NRG1 Type III Activates NF-κB in Schwann Cells

actorize how these homologous ligands can induce these distinct biological outcomes.

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REFERENCES
1. Nickols, J. C., Valentine, W., Kanwal, S., and Carter, B. D. (2003) Nat. Neurosci. 6, 161–167
2. Ghosh, S., May, M. J., and Kopp, E. B. (1998) Annu. Rev. Immunol. 16, 225–260
3. Rayet, B., and Gélinas, C. (1999) Oncogene 18, 6938–6947
4. Mattson, M. P. (2003) Nat. Neurosci. 6, 105–106
5. Yoon, C., Korade, Z., and Carter, B. D. (2008) J. Neurosci. 28, 3738–3746
6. Monuki, E. S., Weinmaster, G., Kuhn, R., and Lemke, G. (1989) Neuron 3, 783–793
7. Poduslo, J. F., Walikonis, R. S., Domec, M. C., Berg, C. T., and Holtzhueter, D. M. (2001) Exp. Cell Res. 284, 143–150
8. Monje, P. V., Athauda, G., and Wood, P. M. (2008) J. Cell Biol. 18393–18401
9. Halama, N., Kulesza, M., and Wistow, G. (1992) PNAS 89, 10867–10871
10. Monje, P. V., Bartlett Bunge, M., and Wood, P. M. (2006) Glia 54, 1491–1497
11. Nave, K. A. (2003) Exp. Cell Res. 290, 109–113
12. Grinspan, J. B., Marchionni, M. A., Reeves, M., Coulalagouli, M., and Scherer, S. S. (1996) J. Neurosci. 16, 6017–6118
13. Salzer, J. L., Williams, A. K., Glaser, L., and Bunge, R. P. (1980) J. Cell Biol. 84, 753–766
14. Michailov, G. V., Sereda, M. W., Brinkmann, B. G., Fischer, T. M., Haug, B., Birchmeier, C., Role, L., Lai, C., Schwab, M. H., and Nave, K. A. (2004) Science 304, 700–703
15. Zanazzi, G., Einheber, S., Westreich, R., Hannon, M. J., Bedell-Hogan, D., Marchionni, M. A., and Salzer, J. L. (2001) J. Cell Biol. 152, 1289–1299
16. Nave, K. A., and Salzer, J. L. (2006) Curr. Opin. Neurobiol. 16, 492–500
17. White, F. V., Caccarini, C., Georgieff, I., Matthieu, J. M., and Costantino-Caccarini, E. (1983) Exp. Cell Res. 148, 183–194
18. Maurel, P., and Salzer, J. L. (2000) J. Cell Biol. 150, 4635–4645
19. Wang, J. Y., Miller, S. J., and Falls, D. L. (2001) J. Biol. Chem. 276, 2841–2851
20. Gentry, J. J., Casaccia-Bonnefil, P., and Carter, B. D. (2000) J. Biol. Chem. 275, 7558–7565
21. Qu, S., Rinehart, C., Wu, H. H., Wang, S. E., Carter, B., Xin, H., Kotlikoff, M., and Arteaga, C. L. (2006) Nature 444, 477–486
22. Palmada, M., Kanwal, S., Rutkoski, N. J., Gustafson-Brown, C., Johnson, R. S., Wisdom, R., and Carter, B. D. (2002) J. Cell Biol. 158, 453–461
23. Garratt, A. N., Britsch, S., and Birchmeier, C. (2000) BioEssays 22, 987–996
24. Garratt, A. N., Voicescu, O., Topilko, P., Charnay, P., and Birchmeier, C. (2000) J. Cell Biol. 148, 1035–1046
25. Leimeiroth, R., Lobos, F., Lüssi, A., Taylor, V., Suter, U., and Sommer, L. (2002) Dev. Biol. 246, 245–258
26. Tavellgia, C., Zanazzi, G., Petrylak, A., Yano, H., Rosenbluth, J., Einheber, S., Xu, X., Esper, R. M., Loeb, J. A., Shragger, P., Chao, M. V., Falls, D. L., Role, L., and Salzer, J. L. (2005) Neuron 47, 681–694
27. Pianetti, S., Arsura, M., Romieu-Mourez, R., Coffey, R. J., and Sonenshein, G. E. (2001) Oncogene 20, 1287–1299
28. Holmes, W. E., Slawkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., and et al. (1992) Science 256, 1205–1210
29. Ogata, T., Iijima, S., Hoshikawa, S., Miura, T., Yamamoto, S., Oda, H., Nakamura, K., and Tanaka, S. (2004) J. Neurosci. 24, 6724–6732
30. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) Nature 401, 82–85
31. Dhawan, P., and Richardson, A. (2002) J. Biol. Chem. 277, 7920–7928
32. Gedey, R., Jin, X. L., Hintzong, O., and Shisler, J. L. (2006) J. Virol. 80, 8676–8685
33. Romashkova, J. A., and Makarov, S. S. (1999) Nature 401, 86–90
34. Tapinos, N., Ohnishi, M., and Rambukkana, A. (2006) Nat. Med. 12, 961–966
35. Baselga, J., and Hammond, L. A. (2002) Oncology 63, Suppl. 1, 6–16
36. Traxler, P., Bold, G., Buchdunger, E., Caravatti, G., Furet, P., Manley, P., O’Reilly, T., Wood, J., and Zimmermann, J. (2001) Med. Res. Rev. 21, 499–512
37. Cabioglu, N., Summy, J., Miller, C., Parikh, N. U., Sahin, A. A., Tuzlali, S., Pumiglia, K., Gallic, G. E., and Price, J. E. (2005) Cancer Res. 65, 6493–6497
38. Kirillova, I., Chaisson, M., and Fausto, N. (1999) Cell Growth Diff. 10, 819–828
39. Lin, Y. Z., Yao, S. Y., Veach, R. A., Torgerson, T. R., and Hawiger, J. (1995) J. Biol. Chem. 270, 14255–14258
40. Cho, H. H., Song, J. S., Yu, J. M., Yu, S. S., Choi, S. J., Kim, D. H., and Jung, J. S. (2008) FEBS Lett. 582, 616–622
41. Einheber, S., Milner, T. A., Giancotti, F., and Salzer, J. L. (1993) J. Cell Biol. 123, 1223–1236
42. Chen, S., Velardez, M. O., Warot, X., Yu, Z. X., Miller, S. J., Cros, D., and Corfas, G. (2006) J. Neurosci. 26, 3079–3086
43. Monje, P. V., Bartlett Bunge, M., and Wood, P. M. (2006) Glia 53, 649–659
44. Monje, P. V., Athauda, G., and Wood, P. M. (2008) J. Biol. Chem. 283, 34087–34100
45. Reddy, S. A., Huang, J. H., and Liao, W. S. (1997) J. Biol. Chem. 272, 29167–29173
46. Sweeney, C., Lai, C., Riese, D. J., 2nd, Diamonti, A. J., Cantley, L. C., and Carraway, K. L. (2000) J. Biol. Chem. 275, 86–90
47. Sweeney, C., Lai, C., Cantley, L. C., and Carraway, K. L. (2000) J. Biol. Chem. 275, 1289–1299
48. Sweeney, C., Fambrough, D., Huard, C., Diamonti, A. J., Lander, E. S., Cantley, L. C., and Carraway, K. L., 3rd. (2001) J. Biol. Chem. 276, 22685–22698
49. Cosgaya, J. M., Chan, J. R., and Shooter, E. M. (2002) Science 298, 1245–1248
50. Naj, A. J., Day, K. C., and Day, M. L. (2008) J. Biol. Chem. 283, 18393–18401