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Synergistic Regulation of Schwann Cell Proliferation by Heregulin and Forskolin

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A peptide corresponding to the epidermal growth factor homology domain of β-heregulin stimulated autophosphorylation of the heregulin receptors erbB2 and erbB3 in Schwann cells and activation of the mitogen-activated protein (MAP) kinases ERK1 and ERK2. Heregulin-dependent activation of PAK65, a component of the stress-activated signaling pathway, ribosomal S6 kinase, and a cyclic AMP (cAMP) response element binding protein (CREB) kinase, identified as p95^{RSK2}, was also observed. Receptor phosphorylation and activation of these kinases in response to heregulin occurred in the absence of forskolin stimulation and were not augmented in cells treated with forskolin, a direct activator of adenylyl cyclase. Schwann cell proliferation in response to heregulin was observed only when the cells were also exposed to an agent that elevates cAMP levels. In the absence of heregulin, elevation of cAMP levels failed to stimulate Schwann cell proliferation. Forskolin significantly enhanced heregulin-stimulated expression of cyclin D and phosphorylation of the retinoblastoma gene product. In cells treated with both heregulin and forskolin there was a sustained accumulation of phospho-CREB, which was not observed in cells treated with either agent alone. Heregulin and forskolin synergistically activated transcription of a cyclin D promoter construct. These results demonstrate that heregulin-stimulated activation of MAP kinase is not sufficient to induce maximal Schwann cell proliferation. Expression of critical cell cycle regulatory proteins and cell division require activation of both heregulin and cAMP-dependent processes.

Myelination of axons by Schwann cells is critical for the proper functioning of the peripheral nervous system. The correct ratio of Schwann cells to axons is achieved during development through a combination of Schwann cell proliferation (26) and programmed cell death (29). Studies with primary cultures of Schwann cells and embryonic sensory neurons have shown that molecular signals that stimulate Schwann cell proliferation are associated with axonal membranes (24, 27, 35).

Several lines of evidence suggest that the axonal Schwann cell mitogen is a member of the heregulin family of growth factors (5, 9, 17, 21). A common structural feature of heregulins is a cysteine-rich domain of approximately 50 amino acids that is homologous to the active domain of epidermal growth factor (EGF) (18). Heregulins stimulate cell proliferation by binding to and activating transmembrane receptor tyrosine kinases with homology to the EGF receptor, called erbB2, erbB3, and erbB4 (10, 25). A synthetic peptide corresponding to the heregulin EGF homology domain is sufficient to mediate binding to erbB receptors (2). Ligand-dependent activation of erbB receptors leads to activation of the mitogen-activated protein (MAP) kinase pathway, which is critical for cell division in many cell types (22).

Schwann cell proliferation can also be stimulated by other polypeptide growth factors (6), including basic fibroblast growth factor and platelet-derived growth factor (PDGF). An unusual feature of the response of Schwann cells to these mitogens is the additional requirement for an agent that raises intracellular cyclic AMP (cAMP) levels in order to produce cell division (5, 6, 12, 23). This is in contrast to most cell types, whose proliferation is inhibited by cAMP (3, 13, 31). cAMP has been reported to be required for expression of PDGF and insulin-like growth factor receptors in Schwann cells (34).

One of the exceptions to the inhibitory effect of cAMP on cell division occurs in dog thyroid cells (14, 16). Elevation of cAMP levels in these cells by treatment with thyroid-stimulating hormone or forskolin induces cell proliferation. This effect occurs in the absence of other mitogens or growth factors. Interestingly, cAMP-stimulated proliferation of these cells does not require activation of the MAP kinase pathway (14) but is dependent on expression of the cell cycle regulatory protein cyclin D (16).

cAMP-dependent regulation is mediated through protein kinase A (PKA). cAMP binds to the regulatory subunit of PKA, causing release of the active catalytic subunit and phosphorylation of target proteins. One of the targets of PKA-mediated phosphorylation is the cAMP response element binding protein, or CREB (19). CREB is a DNA-binding protein that binds to cAMP response elements in the promoters of target genes and, in its phosphorylated form, activates their transcription. CREB kinase activities are also induced by some growth factors, such as insulin and nerve growth factor, via the ras-MAP kinase pathway (7, 36).

In this paper data are presented which demonstrate that β-heregulin stimulates phosphorylation of erbB2 and erbB3 in Schwann cells, activation of the MAP kinase pathway, and transient phosphorylation of CREB. Heregulin-dependent activation of these processes is not sufficient, however, to elicit expression of cyclin D or to stimulate Schwann cell proliferation. Schwann cell proliferation is shown to require long-term costimulation with both heregulin and an activator of adenylyl cyclase.

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MATERIALS AND METHODS

Materials. Monoclonal antibodies that recognize the phosphorylated forms of MEK1 and MEK2 were obtained from New England Biolabs, Inc. Rabbit polyclonal antibodies against CREB and phospho-CREB and mouse monoclonal antibodies against erbB2, erbB4, and $p95^{RSK2}$ were from Upstate Biotechnology, Inc. Monoclonal anti-cyclin D, anti-ribosomal S6 kinase, and polyclonal anti-erbB3 antibodies were from Santa Cruz Laboratories. Anti-phospo-MAP kinase antibodies were from New England Biolabs. [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Dupont-NEN. [*methyl*-^{3}H]thymidine (76 Ci/mmol) was purchased from Amersham Life Sciences. Myelin basic protein was obtained from GIBCO-BRL. CREBtide, a peptide containing the phosphorylation site of CREB (KRREILSRRPSYRK), and a peptide corresponding to the EGF homology domain of β-heregulins (SHLVKCAEKEKTFCVNGGECFMVKDL SNPSRYLCKCPNEFTGDRCQNYVM) were synthesized in the Weis Center for Research Molecular Biology Core Laboratory. The latter peptide was purified as described previously (2). Forskolin, insulin, and transferrin were from Sigma Chemical Co. Other culture media components were from GIBCO-BRL.

Cell culture. Schwann cells were isolated from the sciatic nerves of newborn rats as described previously (4). For routine culture the cells were grown on poly-L-lysine-coated tissue culture dishes in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum and 2 μM forskolin. The cultures contained >95% Schwann cells as indicated by staining with cell-specific antibodies. For the experiments described in this paper, the cells were used between passage 2 and passage 5.

Cell proliferation assays. Confluent cultures of Schwann cells, cultured as described above, were trypsinized and replated in poly-L-lysine-coated 96-well plates at a density of 50,000 cells/cm^2. The cells were allowed to attach overnight in DME–10% fetal bovine serum containing 2 μM forskolin. The next day the medium was switched to serum-free medium (DME–Ham's F-12, 1:1, supplemented with 0.1 mg of transferrin and 0.1 μg of insulin per ml) containing mitogens and forskolin as indicated in Results. After 6 days the cell numbers were determined by the CellTiter 96 AQueous assay (Promega). The absorbance at 490 nm was measured with a Spectramax 250 microplate spectrophotometer.

In some experiments cell proliferation was assayed by measuring the incorporation of ^{3}H-thymidine. The cells were plated at a density of 50,000 cells/cm^2 in poly-L-lysine-coated 24-well plates and incubated overnight in DME–10% fetal bovine serum. The cells were then incubated in serum-free medium without mitogens or forskolin for 24 h. The cells were switched to serum-free medium without or with heregulin and/or forskolin, as indicated in Results. The medium was supplemented with 1 μCi of [^{3}H]thymidine per ml. The cells were lysed at various times after mitogen stimulation (48 h in most experiments). Aliquots were precipitated with trichloroacetic acid. The precipitates were collected on glass fiber filters and counted in a liquid scintillation counter.

Receptor phosphorylation assays. Schwann cells were trypsinized and replated at a density of 50,000 cells/cm^2 in poly-L-lysine-coated culture plates (60-mm diameter) and allowed to attach overnight in DME–10% fetal bovine serum. The cells were incubated for 24 h in serum-free medium without or with forskolin. Fresh medium containing heregulin and/or forskolin was added, and the cells were incubated at 37°C for various times (see Results). The cells were lysed in immunoprecipitation buffer (0.5% Nonidet P-40; 0.5% deoxycholate; 0.1% sodium dodecyl sulfate [SDS]; 50 mM Tris-HCl, pH 7.5). Aliquots were immunoprecipitated with anti-erbB2, anti-erbB3, or anti-erbB4 antibodies. Immune complexes were precipitated by addition of protein A-Sepharose beads coated with anti-rabbit immunoglobulin G. The proteins were resolved by SDS-gel electrophoresis, transferred to Immobilon membranes, blocked in 5% nonfat milk in 50 mM Tris-HCl (pH 7.5) and 100 mM NaCl, and stained with antiphosphotyrosine antibodies. Bound antibodies were detected by enhanced chemiluminescence.

Kinase assays. Schwann cells were trypsinized and replated in poly-L-lysine-coated six-well plates (35-mm diameter per well) and were allowed to attach overnight in DME–10% fetal bovine serum. The cells were then incubated in serum-free medium with various concentrations of forskolin for 24 h. The cells were stimulated with heregulin peptide for various times in the absence or presence of forskolin, as indicated in Results. The cells were rinsed quickly with cold phosphate-buffered saline and then lysed in 100 μl of kinase lysis buffer (20 mM Tris-HCl, pH 7.5; 1% Triton X-100; 20 mM NaF; 2 mM EDTA; 0.1 mM Na orthovanadate; 1 mM dithiothreitol [DTT]; 2 mM 4-(2-aminoethyl)benzene sulfonyl flouride (AEBSF); 10 mM benzamidine; and 1 μg each of aprotinin and leupeptin per ml) per well. The cells were scraped into Microfuge tubes, lysed by free thawing, and shaken on a rotator for 10 min at 4°C. Protein levels were determined with the Bio-Rad assay kit.

MAP kinase activity was measured by a modification of the in-gel method described previously (11). Equal amounts of protein from cell extracts were separated on SDS–10% polyacrylamide gels that contained 0.1 mg of myelin basic protein per ml. After electrophoresis the gels were incubated sequentially in 50 mM Tris-HCl (pH 8), 20% isopropanol (twice for 30 min each), 50 mM Tris-HCl (pH 8), 5 mM 2-mercaptoethanol (once for 60 min), 50 mM Tris-HCl (pH 8), and 6 M guanidine (twice for 30 min each). Proteins in the gel were renatured by incubation for 16 h at 4°C in 50 mM Tris-HCl (pH 8)–0.04% Tween 20. The gels were then incubated for 1 h at room temperature in 40 mM Na HEPES (pH 8), 2 mM DTT, and 10 mM $MgCl_2$. Kinase assays were carried out for 1.5 h at 37°C in a mixture of 40 mM Na HEPES (pH 8), 2 mM DTT, 10 mM $MgCl_2$, and 0.5 mM EGTA containing 10 μCi of [γ-^{32}P]ATP (0.1 mM) per ml. Reactions were terminated by placing the gels in 5% trichloroacetic acid and 1% sodium pyrophosphate. The gels were washed several times in this solution to remove unreacted radioactivity. The gels were dried and exposed on Kodak X-Omat film with an intensifying screen. Radioactivity was quantitated by PhosphorImager analysis (Molecular Dynamics). In-gel PAK65 (30) and CREB kinase (7) assays were performed in a similar manner, except that the gels contained 0.2 mg of histone H1 or 0.2 mg of CREBtide per ml as the substrate. For CREB kinase assays the reaction buffer was 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], (pH 7.2), 10 mM $MgCl_2$, 2 mM DTT, and 20 μCi of [γ-^{32}P]ATP (0.1 mM).

MAP kinase activation was also assayed by immunoblot analysis of aliquots of cell lysates with antibodies that specifically recognize the phosphorylated forms of ERK1 and ERK2.

Cyclin D3 promoter-reporter vector construction and transfection. An 835-bp DNA segment immediately upstream of the rat cyclin D3 transcription start site was obtained by PCR amplification with specific primers based on the published gene sequence (37) and rat genomic DNA (Clontech). The amplified product was cloned into plasmid pCR3.1 (Clontech). The DNA sequence of the insert (data not shown) was identical to the rat cyclin D3 5′ flanking sequence reported previously (37). The insert was excised by digestion with *Xho*I and *Nhe*I and subcloned into the multiple cloning site of the plasmid pGL3-basic (Promega), immediately upstream of the luciferase coding region. Correct insertion of the cyclin D3 promoter sequence was verified by DNA sequence analysis.

Promoter activity was determined in transiently transfected Schwann cells. The cells were plated in 24-well culture dishes and transfected with 0.2 μg of cyclin D reporter plasmid for 3 h by using Lipofectamine-Plus in serum-free OptiMEM (Life Technologies, Inc.). Following transfection the cells were allowed to recover overnight in DME–10% fetal bovine serum. The cells were then incubated for 24 h in serum-free medium. During this period the medium was changed three times. This was necessary to remove residual serum components and to decrease the basal cyclin D reporter activity. The cells were then stimulated with heregulin and/or forskolin as described in Results. Forty-eight hours later the cells were lysed. Aliquots were used for assays of luciferase activity with the Promega assay kit and an EG & G Berthold luminometer.

RESULTS

Mitogenic activity of β-heregulin requires elevation of cAMP. Stimulation of Schwann cell proliferation with purified mitogens, such as basic fibroblast growth factor or PDGF, requires costimulation with an agent that results in elevation of cAMP levels (6, 23). Experiments were carried out to determine whether elevation of cAMP levels was required for heregulin-dependent mitogenic activity. Schwann cells were cultured in the absence or presence of a synthetic peptide corresponding to the EGF homology domain of β-heregulin (2). As shown in Fig. 1A, heregulin peptide failed to produce a significant increase in the number of cells present after 6 days, compared with cultures incubated in serum-free medium without growth factor. In contrast, treatment of Schwann cells with heregulin in the presence of forskolin, a direct activator of adenylyl cyclase, produced a significant increase in cell numbers. Forskolin at concentrations of 0.2 μM and 2 μM produced similar responses. In the absence of heregulin, forskolin had no effect on cell numbers. The requirement for forskolin could not be overcome by increasing the heregulin concentration (Fig. 1B). The effects of heregulin on cell numbers were not due to increased survival. The cell numbers determined after 6 days represented an increase over the numbers that had been plated initially. Moreover, under the conditions used in this assay, no significant loss of Schwann cells was observed in the absence of exogenous growth factors (data not shown).

Fetal bovine serum in the absence of other mitogens also stimulated Schwann cell proliferation, although to a lesser degree than heregulin. Interestingly, the mitogenic activity of serum was not dependent on forskolin. Addition of heregulin to medium containing 10% fetal bovine serum produced an increase in cell numbers that was greater than that produced by either agent alone. Similar to what was observed in serum-free medium, the heregulin-dependent stimulation of proliferation in the presence of serum required forskolin.

A similar effect of forskolin on heregulin-dependent mito-

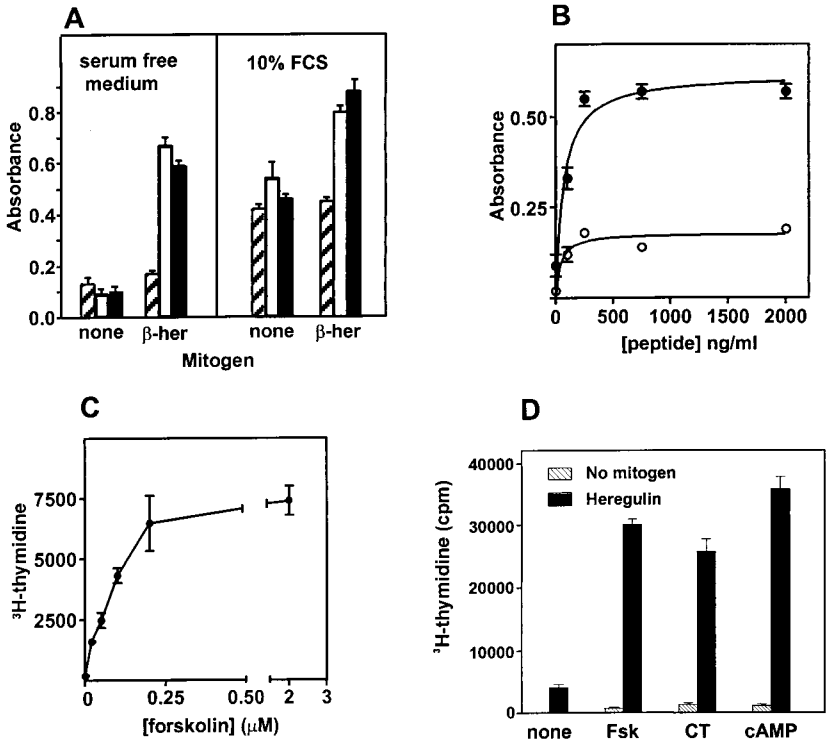


FIG. 1. Effect of forskolin on heregulin-dependent Schwann cell proliferation. (A) Schwann cells were incubated for 6 days in serum-free medium or DME–10% fetal calf serum (FCS) in the absence (none) or presence of 250 ng of heregulin peptide (β-her) per ml, without forskolin (hatched bars) or with 0.2 μM (open bars) or 2 μM (solid bars) forskolin. The values shown are the means ± standard errors of the mean for eight wells. This experiment was carried out three times with similar results. (B) Schwann cells were incubated for 6 days in serum-free medium without (open circles) or with (filled circles) 2 μM forskolin and the indicated concentration of heregulin peptide. Values shown are means ± standard errors of the mean for eight wells. (C) Schwann cells were made quiescent by incubation for 24 h in serum-free medium and then were switched to serum-free medium containing 250 ng of heregulin peptide per ml and the indicated concentrations of forskolin plus [^{3}H]thymidine. The cells were incubated for 48 h and incorporation of [^{3}H]thymidine was determined as described in Materials and Methods. Values shown are means ± standard errors of the mean for eight wells. (D) Schwann cells were treated as described for C, except that serum-free medium without (hatched bars) or with (solid bars) heregulin was supplemented with 2 μM forskolin (Fsk), cholera toxin (CT), or 8-Br-cAMP (cAMP).

genic activity was observed when cell proliferation was assayed by [^{3}H]thymidine incorporation. Addition of forskolin to serum-free medium containing heregulin increased [^{3}H]thymidine incorporation nearly 40-fold over what was measured in the absence of forskolin (Fig. 1C). In the absence of heregulin, forskolin did not stimulate incorporation of [^{3}H]thymidine (data not shown). Maximal stimulation of heregulin-dependent DNA synthesis was observed at forskolin concentrations of 0.2 μM or greater (Fig. 1C). Similar stimulation of heregulin-dependent [^{3}H]thymidine incorporation was observed when cholera toxin or 8-Br-cAMP was used in place of forskolin (Fig. 1D). Cholera toxin directly activates the α subunit of G_s, the heterotrimeric GTP-binding protein responsible for stimulation of adenylyl cyclase. 8-Br-cAMP is a cell-permeative cAMP analogue. These results suggest that effects of forskolin on Schwann cell proliferation are dependent on its ability to activate adenylyl cyclase.

Phosphorylation of erbB receptors and activation of MAP kinase by heregulin. Heregulin growth factors stimulate cell proliferation by binding to and activating erbB receptor kinases (10, 25). Consistent with previous reports (29, 32), stimulation of Schwann cells with heregulin resulted in tyrosine phosphorylation of erbB2 and erbB3 (Fig. 2A). Phosphorylation of erbB4 was not detected (data not shown).

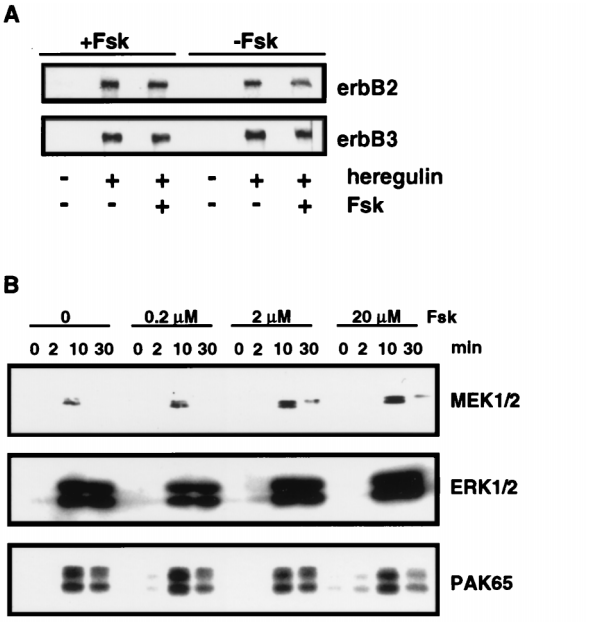
To determine whether cAMP affected heregulin receptor expression or heregulin-dependent receptor activation, Schwann cells were grown for 48 h in serum-free medium in the absence or presence of 2 μM forskolin and then stimulated with heregulin in the absence or presence of 2 μM forskolin. As shown in Fig. 2A, phosphorylation of erbB2 and erbB3 in response to heregulin was observed in the absence of forskolin. Neither long-term nor acute treatment with forskolin had any effect on heregulin-stimulated tyrosine phosphorylation of the receptors.

Heregulins have been reported to activate MAP kinase in Schwann cells (12). Stimulation of Schwann cells with heregulin peptide caused a rapid, sustained activation of the MAP kinase pathway. Heregulin activated the MAP kinase kinases MEK1 and MEK2 and the 44- and 42-kDa forms of MAP kinase (ERK 1 and ERK2) (Fig. 2B).

PAK65 is a kinase that is a component of the stress-activated signaling pathway in some cells (1, 38). As shown in Fig. 2B, PAK65 was also activated in response to heregulin stimulation of Schwann cells. Activation of the 70-kDa ribosomal S6 kinase, $p70^{RSK}$ (Fig. 2C), and $p95^{RSK2}$ (see below), a downstream target of MAP kinase (36), was also observed in heregulin-treated cells.

Forskolin had no effect on heregulin-dependent activation of MEK, MAP kinase, PAK65 (Fig. 2B), $p70^{RSK}$ (Fig. 2C), or $p95^{RSK2}$ (see below). These results demonstrate that stimulation of proliferation by forskolin does not result from induction of heregulin receptor expression, from changes in heregulin-dependent receptor activation, or from coupling to downstream kinase pathways.

Schwann cell proliferation requires long-term simultaneous exposure to heregulin and forskolin. When Schwann cells were



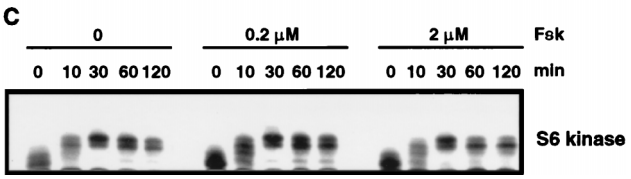


FIG. 2. Heregulin-dependent activation of erbB receptors and signaling kinases in Schwann cells. (A) Schwann cells were incubated for 48 h in serum-free medium without forskolin (−Fsk) or with 2 μM forskolin (+Fsk). The cells were then stimulated with 250 ng of heregulin peptide per ml in medium that lacked (−) or contained (+) 2 μM forskolin. After 15 min the cells were lysed and aliquots of control and heregulin-stimulated cells were immunoprecipitated with antibodies to erbB2 or erbB3 and subjected to immunoblot analysis with antiphosphotyrosine antibodies. (B) Schwann cells were incubated for 24 h in serum-free medium containing the indicated concentrations of forskolin (Fsk). Heregulin peptide (250 ng/ml) was added and the cells were lysed at the indicated times after heregulin addition. Aliquots of the lysates were assayed for MEK activation by immunoblot analysis with anti-phospho-MEK1,2 antibody (upper panel) and for MAP kinase (middle panel) and PAK65 (lower panel) activation by in-gel kinase assays. (C) Schwann cells were treated as described for B. Aliquots of cell lysates were subjected to immunoblot analysis with anti-$p70^{RSK}$ antibodies.

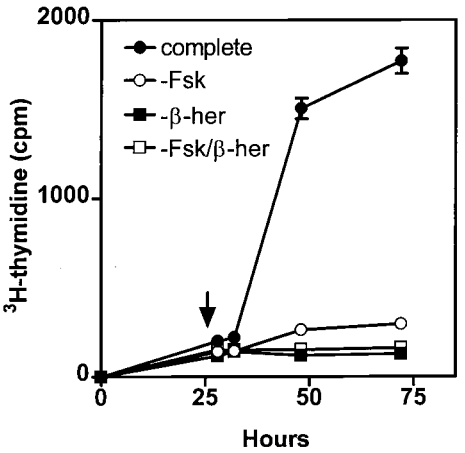


FIG. 3. Schwann cell proliferation requires continuous exposure to heregulin and forskolin. Schwann cells were replated and incubated in serum-free medium for 24 h and then switched (at time zero) to serum-free medium containing 250 ng of heregulin peptide per ml and 2 μM forskolin. After 24 h (arrow) the medium was changed and the cells were incubated for an additional 48 h in serum-free medium supplemented with both heregulin and forskolin (complete) heregulin only (−Fsk), forskolin only (−β-her) or neither (−Fsk/β-her). Incorporation of [^{3}H]thymidine was assayed as described in Materials and Methods.

incubated for 24 h in serum-free medium without mitogens and then switched to medium containing heregulin and forskolin, a large increase in DNA synthesis occurred between 32 and 48 h after initiation of heregulin-forskolin stimulation (Fig. 3). These results suggest that incubation in serum-free medium arrests the Schwann cells in a G_0-like state and that progression to S phase occurs in approximately 32 h. This is consistent with earlier measurements of the time course of DNA synthesis in Schwann cells (12). When the cells were incubated in medium containing heregulin and forskolin for 24 h and then switched to medium containing only heregulin or forskolin, entry into S phase did not occur (Fig. 3). Preincubation of Schwann cells in medium containing forskolin for 24 h, followed by incubation in medium with heregulin (without forskolin), also failed to stimulate Schwann cell proliferation (data not shown). These results demonstrate that long-term simultaneous exposure to forskolin and heregulin is needed to stimulate Schwann cell division.

Heregulin-dependent expression of cyclin D is potentiated by forskolin. Mitogen-dependent stimulation of cell proliferation requires expression of the cell cycle regulatory protein cyclin D (28). Schwann cells were incubated in serum-free medium for 24 h and then stimulated with heregulin in the absence or presence of forskolin. The accumulation of cyclin D was determined by immunoblot analysis. As shown in Fig. 4A, heregulin weakly stimulated cyclin D accumulation in Schwann cells. Incubation in medium with heregulin and forskolin, however, produced a significantly higher level of cyclin D accumulation than incubation in medium lacking forskolin (Fig. 4A). Treatment with forskolin alone failed to induce cyclin D accumulation (data not shown). In cells treated with heregulin and forskolin cyclin D was detected after a lag period of several hours, and it peaked approximately 11 h after initiation of β-heregulin stimulation (Fig. 4A). Cyclin D levels remained elevated for at least 48 h after stimulation with heregulin and forskolin (data not shown).

Cell division in many cells requires phosphorylation of the retinoblastoma gene product, pRb (33). Phosphorylation of pRb is accomplished by the cyclin-dependent kinases cdk4 and cdk6. Activity of these kinases is stimulated by association with cyclin D (28). As shown in Fig. 4B, exposure of quiescent Schwann cells to forskolin or heregulin failed to elicit pRb phosphorylation, as determined by the lack of a shift in the electrophoretic mobility of the protein. In contrast, treatment of Schwann cells with heregulin and forskolin resulted in a distinct reduction in mobility of pRb. Steady-state levels of pRb also appeared to be increased in cells exposed to both heregulin and forskolin. pRb phosphorylation in response to heregulin and forskolin was detected after a lag period of 18 h and persisted until at least 48 h.

Heregulin- and forskolin-dependent CREB phosphorylation. The results presented above suggest that stimulation with both heregulin and forskolin is required for high-level expression of cyclin D. The best-characterized mechanism for cAMP-dependent regulation of gene expression involves the PKA-

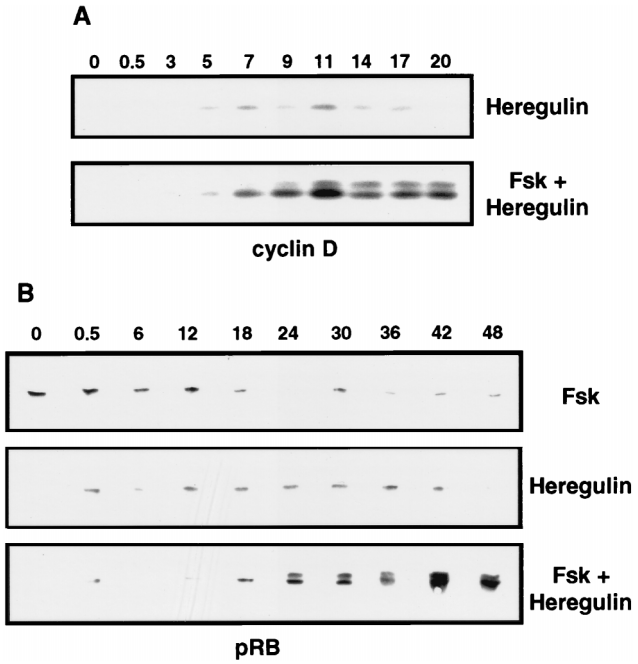


FIG. 4. Forskolin potentiates heregulin-stimulated expression of cyclin D and pRb phosphorylation. Schwann cells were incubated for 24 h in serum-free medium and then stimulated with 2 μM forskolin (Fsk), 250 ng of heregulin peptide per ml, or heregulin plus 2 μM forskolin. At the indicated times (in hours) after addition of these agents, the cells were lysed. Aliquots were subjected to immunoblot analysis and stained with antibodies to cyclin D (A) or pRb (B).

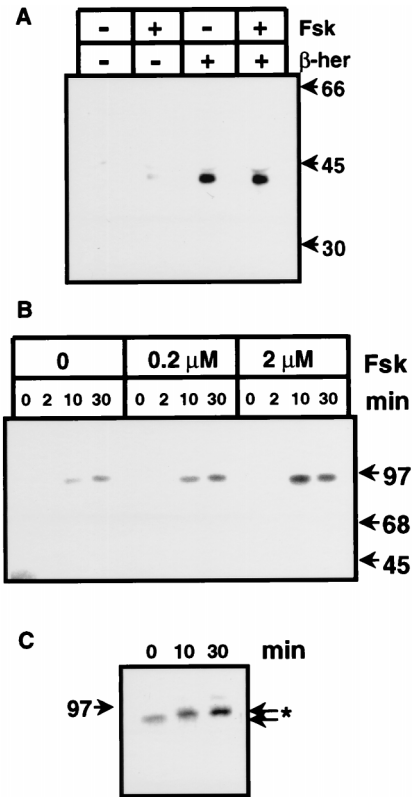


FIG. 5. Identification of heregulin-stimulated CREB kinase activity. (A) Schwann cells were incubated for 24 h in serum-free medium and then stimulated with 2 μM forskolin (Fsk) and/or 250 ng of heregulin peptide (β-her) per ml. After 30 min the cells were lysed and aliquots were subjected to immunoblot analysis with anti-phospho-CREB antibodies. (B) Schwann cells were incubated for 48 h in serum-free medium without forskolin or with 0.2 μM or 2 μM forskolin. Heregulin peptide (250 ng/ml) was added and the cells were lysed at the indicated times after heregulin addition. Aliquots of control and heregulin-stimulated cells were used to assay CREB kinase activity by means of an in-gel assay. The region of the gel below 45 kDa is not shown. (C) Schwann cells were incubated for 24 h in serum-free medium and then stimulated with 250 ng of heregulin peptide per ml. At the indicated times (in minutes) the cells were lysed, and aliquots were subjected to immunoblot analysis with anti-p95^{RSK2} antibodies. The arrows on the right indicate positions of migration of p95^{RSK2} before and after (*) stimulation with heregulin. Positions of migration of molecular mass markers (in kilodaltons) are indicated in all panels.

mediated phosphorylation of CREB (19). CREB activation was assayed in Schwann cells by immunoblot analysis with an antibody that recognizes CREB that is phosphorylated at the site (serine 133), shown to be critical for transcriptional activation. Phospho-CREB was not detected in quiescent Schwann cells incubated in serum-free medium. Stimulation with 2 μM forskolin produced a modest increase in phospho-CREB (Fig. 5A). Stimulation of the cells with heregulin produced a rapid and robust increase in phospho-CREB (Fig. 5A). When assayed at times ranging from 15 to 40 min after stimulation, heregulin was more effective than forskolin in stimulating CREB phosphorylation. At these times, forskolin had no apparent effect on heregulin-dependent CREB phosphorylation.

An in-gel kinase assay revealed two CREB kinase activities in Schwann cells. One of these corresponded to a polypeptide of 42 kDa. The CREB kinase activity of this protein was blocked by H-89, a specific PKA inhibitor (data not shown). These results identify this kinase as the catalytic subunit of PKA. The other CREB kinase activity was associated with a polypeptide of 95 kDa (Fig. 5B). CREB kinase activity of this protein was not observed in the absence of heregulin but was stimulated rapidly following heregulin treatment. Heregulin-dependent stimulation of the 95-kDa CREB kinase activity occurred in the absence of forskolin. Forskolin alone had no effect on the activity of this kinase but appeared to cause an increase in its activity following heregulin stimulation of the cells.

p95^{RSK2} is a protein that has been shown to possess CREB kinase activity and to be a substrate for phosphorylation by MAP kinase (36). p95^{RSK2} was identified by immunoblot analysis in Schwann cell extracts. Moreover, heregulin stimulation of the cells produced a reduction in the electrophoretic mobility of p95^{RSK2} (Fig. 5C), consistent with its modification by phosphorylation. Taken together, these results strongly suggest that the heregulin-stimulated Schwann cell CREB kinase is p95^{RSK2}.

The effects of long-term stimulation with heregulin and forskolin on CREB phosphorylation were also examined. As shown in Fig. 6B, heregulin-stimulated CREB phosphorylation was transient and did not coincide temporally with heregulin-forskolin-stimulated expression of cyclin D (Fig. 4A). In contrast, continuous exposure to both heregulin and forskolin resulted in a prolonged phosphorylation of CREB (Fig. 6C). Under these conditions there appeared to be a biphasic response. This sustained increase in CREB phosphorylation was not observed in cells treated with forskolin alone (Fig. 6A).

Heregulin and forskolin synergistically promote activation of cyclin D transcription. To examine more directly the effects of heregulin and forskolin on cyclin D expression, a reporter construct was generated in which transcription of the luciferase gene was regulated by an 835-bp DNA sequence corresponding to the 5′ flanking region of the rat cyclin D3 gene (37).

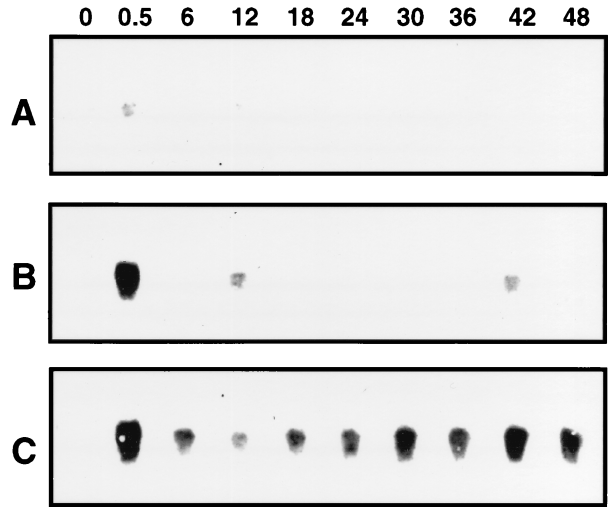


FIG. 6. Time course of heregulin- and forskolin-dependent CREB phosphorylation. Schwann cells were incubated for 24 h in serum-free medium and then stimulated with 2 μM forskolin (A), 250 ng of heregulin peptide per ml (B), or forskolin plus heregulin (C). Cells were lysed at the indicated times (in hours) after stimulation. Aliquots were subjected to immunoblot analysis with anti-phospho-CREB antibodies.

Schwann cells were transiently transfected with this reporter vector and then incubated in serum-free medium alone or in medium supplemented with heregulin, forskolin, or both agents. Figure 7 shows the levels of luciferase activity measured in lysates prepared at 48 h after initiation of these treatments. In the absence of heregulin and forskolin a low level of luciferase activity was present in the cells. Forskolin treatment failed to increase luciferase activity and in most experiments produced a slight decrease. Heregulin treatment increased luciferase activity five- to eightfold (data from three separate experiments). Incubation in medium with heregulin and forskolin produced an even greater increase in luciferase activity. Forskolin increased luciferase activity by an average of 3.7-fold (±0.9 in four experiments) over the activity present in cells treated with heregulin alone. These results demonstrate that heregulin and forskolin synergistically activate transcription of the cyclin D3 gene in Schwann cells.

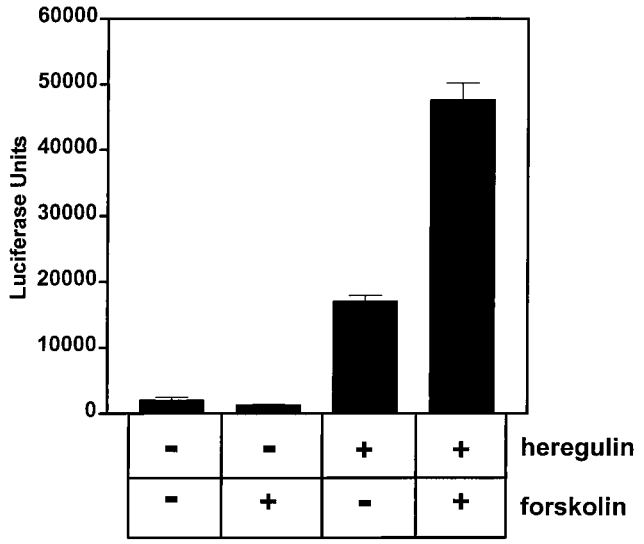


FIG. 7. Synergistic activation of cyclin D3 promoter by heregulin and forskolin. Schwann cells were transiently transfected with the rat cyclin D3 reporter vector as described in Materials and Methods. The cells were made quiescent by incubation in serum-free medium for 24 h and then switched to medium containing 250 ng of heregulin per ml and/or 2 μM forskolin. After 48 h the cells were lysed and aliquots were used to assay luciferase activity. The values shown are means ± standard deviations of triplicate values. The graph shows representative data from one of three independent experiments that produced essentially identical results.

DISCUSSION

The results presented in this paper demonstrate that activation of both heregulin and cAMP-dependent pathways is needed to stimulate proliferation of rat Schwann cells. Activation of cAMP-dependent pathways appeared to have no effect on heregulin receptor autophosphorylation or on coupling to proximal downstream signaling pathways. In spite of this, exposure to both heregulin and forskolin was needed to achieve sustained, high-level cyclin D expression and hyperphosphorylation of pRb. Experiments with a luciferase reporter vector linked to a cyclin D promoter demonstrated that heregulin and forskolin, a direct activator of adenylyl cyclase, activated cyclin D transcription in a synergistic fashion. These findings demonstrate a novel mechanism for cAMP-dependent regulation of heregulin-stimulated Schwann cell proliferation. This mechanism is distinct from that demonstrated previously for PDGF. Forskolin has been reported to be required for expression of PDGF receptors by Schwann cells (34).

Heregulin activated a number of distinct signaling pathways in Schwann cells. Heregulin stimulation led to autophosphorylation of erbB2 and erbB3. At least some of these phosphorylated receptors were present in heteromeric complexes containing both receptor molecules, as indicated by coimmunoprecipitation experiments (27a). This is consistent with the reported lack of a functional kinase domain in erbB3 (8). Receptor activation led to the stimulation of the MEK-MAP kinase pathway, PAK65, a component of the stress-activated signaling pathway (1, 38), $p70^{RSK}$, and $p95^{RSK2}$. Activation of the last kinase was inhibited by treatment with the MEK inhibitor PD98057, consistent with earlier reports demonstrating that $p95^{RSK2}$ is a target for phosphorylation and activation by MAP kinase (36). In-gel kinase assays indicated that this enzyme was also responsible for the heregulin-activated CREB kinase activity that was observed. In PC12 and other cells $p95^{RSK2}$ has been shown to function as an insulin- and nerve growth factor-activated CREB kinase (36). In Schwann cells, activation of this kinase by heregulin (in the absence of forskolin) was transient. This correlated with a transient increase in accumulation of phospho-CREB. A somewhat paradoxical finding was that heregulin was a better stimulus for CREB phosphorylation than forskolin, at least at concentrations that were sufficient to fully potentiate heregulin-dependent proliferation.

The observation that long-term exposure to heregulin and forskolin was required to achieve Schwann cell proliferation suggests a requirement for a change in gene expression. The ability of heregulin and forskolin to produce sustained cyclin D accumulation and Schwann cell division correlated with the maintenance of a high level of phospho-CREB, the transcriptionally activated form of this DNA-binding protein. The observed correlation between sustained CREB phosphorylation and cyclin D expression is consistent with the presence of a CRE site in the cyclin D3 promoter. This site has been shown to be functional in transcriptional activation in lymphoma cells (37).

The mechanism by which combined heregulin and forskolin

treatment leads to sustained CREB phosphorylation is not known. The principal CREB kinase in Schwann cells appears to be $p95^{RSK2}$, which is activated by heregulin via MAP kinase. Forskolin, at concentrations that produced maximal stimulation of cell proliferation (when added with heregulin), only weakly stimulated CREB phosphorylation. This result suggests that forskolin-dependent activation of PKA is not an important pathway for CREB phosphorylation in Schwann cells. If forskolin is not activating the CREB kinase, then what is its role in this process? Several mechanisms could account for the synergistic effect of forskolin and heregulin on phospho-CREB accumulation. One of these is cAMP-mediated inhibition of phospho-CREB phosphatase activity. Indirect evidence for this mechanism comes from the preliminary finding that treatment of Schwann cells with a phosphatase inhibitor increases cyclin D transcription in cells stimulated with heregulin (27a). Other possible mechanisms include forskolin-dependent activation of a kinase that is distinct from $p95^{RSK2}$ or PKA and PKA-dependent activation of $p95^{RSK2}$. Additional experiments will be required to resolve these questions.

cAMP is an important regulator of Schwann cell phenotype (6, 12, 20, 23). Surprisingly, there is little or no information on the physiological mechanisms that regulate adenylyl cyclase activity in Schwann cells. Schwann cells respond to isoproterenol to produce a transient elevation of cAMP levels (3a). The β-adrenergic agonist isoproterenol cannot replace forskolin in promoting heregulin-dependent Schwann cell proliferation, however. In contrast, cholera toxin and 8-Br-cAMP produced Schwann cell proliferation to levels comparable to forskolin. An important difference between these agents and isoproterenol is their ability to generate a sustained elevation of cAMP levels. The β-adrenergic receptor is subject to rapid downregulation (15) and attenuation of adenylyl cyclase activation.

It is important to note that nerve cell-stimulated Schwann cell proliferation does not require an exogenous cAMP-elevating cofactor (26). This is in contrast with virtually all purified mitogens, which are inactive in the absence of such a cofactor. There is convincing evidence that neuronal mitogen is a heregulin (5, 9, 21). This suggests, therefore, that nerve cells also produce an agent that functions as a cAMP agonist. The nature and identity of this factor are not known.

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REFERENCES

1. **Bagrodia, S., B. Derijard, R. J. Davis, and R. A. Cerione.** 1995. Cdc42 and PAK-mediated signaling lead to jun kinase and p38 mitogen-activated protein kinase activation. J. Biol. Chem. **270:**27995–27998.
2. **Barbacci, E. G., B. C. Guarino, J. G. Stroh, D. H. Singleton, K. J. Rosnack, J. D. Moyer, and G. C. Andrews.** 1995. The structural basis for the specificity of epidermal growth factor and heregulin binding. J. Biol. Chem. **270:**9585–9589.
3. **Barlat, I., B. Henglein, A. Plet, N. Lamb, A. Fernandez, F. McKenzie, J. Pouyssegur, A. Vie, and J. M. Blanchard.** 1995. TGF-beta1 and cAMP attenuate cyclin A gene transcription via a cAMP responsive element through independent pathways. Oncogene **11:**1309–1318.

3a. **Bendt, K. M., M. Rahmatullah, and D. J. Carey.** Unpublished data.

4. **Carey, D. J., and R. C. Stahl.** 1990. Identification of a lipid-anchored heparan sulfate proteoglycan in Schwann cells. J. Cell. Biol. **111:**2053–2062.
5. **Dong, Z., A. Brennan, N. Liu, Y. Yarden, G. Lefkowitz, R. Mirsky, and K. R. Jessen.** 1995. Neu differentiation factor is a neuron-glia signal and regulates survival, proliferation, and maturation of rat Schwann cell precursors. Neuron **15:**585–596.
6. **Eccleston, P. A.** 1992. Regulation of Schwann cell proliferation: mechanisms involved in peripheral nerve development. Exp. Cell Res. **199:**1–9.
7. **Ginty, D. D., A. Bonni, and M. E. Greenberg.** 1994. Nerve growth factor activates a ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB. Cell **77:**713–725.
8. **Guy, P. M., J. V. Platko, L. C. Cantley, R. A. Cerione, and K. L. Carraway, III.** 1994. Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity. Proc. Natl. Acad. Sci. USA **91:**8132–8136.
9. **Ho, W.-H., M. P. Armanini, A. Nuijens, H. S. Phillips, and P. L. Osheroff.** 1995. Sensory and motor neuron-derived factor: a novel heregulin variant highly expressed in sensory and motor neurons. J. Biol. Chem. **270:**14523–14532.
10. **Holmes, W. E., M. X. Sliwkowski, R. W. Akita, W. J. Henzel, J. Lee, J. W. Park, D. Yansura, N. Abadi, H. Raab, G. D. Lewis, H. M. Shepard, W.-J. Kuang, W. I. Wood, D. V. Goeddel, and R. L. Vandlen.** 1992. Identification of heregulin, a specific activator of p185erbB2. Science **256:**1205–1210.
11. **Kamesita, I., and H. Fujisawa.** 1989. A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate gel. Anal. Biochem. **183:**139–143.
12. **Kim, H. A., J. E. DeClue, and N. Ratner.** 1997. cAMP-dependent protein kinase A is required for Schwann cell growth: interactions between the cAMP and neuregulin/tyrosine kinase pathways. J. Neurosci. Res. **49:**236–247.
13. **L'Allemain, G., J. N. Lavoie, V. Baldin, and J. Pouyssegur.** 1997. Cyclin D1 expression is a major target of the cAMP-induced inhibition of cell cycle entry in fibroblasts. Oncogene **14:**1981–1990.
14. **Lamy, F., F. Wilkin, M. Baptist, J. Posada, P. P. Roger, and J. E. Dumont.** 1993. Phosphorylation of mitogen activated protein kinases is involved in the epidermal growth factor and phorbol ester, but not in the thyrotropin/cAMP, thyroid mitogenic pathway. J. Biol. Chem. **268:**8398–8401.
15. **Lohse, M. J., J. L. Benovic, M. G. Caron, and R. J. Lefkowitz.** 1990. Multiple pathways of rapid β_2-adrenergic receptor desensitization: delineation with specific inhibitors. J. Biol. Chem. **265:**3202–3211.
16. **Lukas, J., J. Bartkova, and J. Bartek.** 1996. Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D–cyclin-dependent kinase–pRb-controlled G_1 checkpoint. Mol. Cell. Biol. **16:**6917–6925.
17. **Marchionni, M. A., A. D. J. Goodearl, M. S. Chen, O. Bermingham-Mcdonogh, C. Kirk, M. Hendricks, F. Danehy, D. Misumi, J. Sudhalter, K. Kobayashi, D. Wroblewski, C. Lynch, M. Baldassare, I. Hiles, J. B. Davis, J. J. Hsuan, N. F. Totty, M. Otsu, R. N. McBurney, M. D. Waterfield, P. Stroobant, and D. Gwynne.** 1993. Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature (London) **362:**312–318.
18. **Meyer, D., T. Yamaai, A. Garratt, E. Reithmacher-Sonnenberg, D. Kane, L. E. Theill, and C. Birchmeier.** 1997. Isoform-specific expression and function of neuregulin. Development **124:**3575–3586.
19. **Montminy, M.** 1997. Transcriptional regulation by cyclic AMP. Annu. Rev. Biochem. **66:**807–822.
20. **Morgan, L., K. R. Jessen, and R. Mirsky.** 1991. The effects of cAMP on differentiation of cultured Schwann cells: progression from an early phenotype ($O4^+$) to a myelin phenotype (Po^+, $GFAP^-$, $N\text{-}CAM^-$, NGF-Receptor$^-$) depends on growth inhibition. J. Cell Biol. **112:**457–467.
21. **Morissey, T. K., A. D. O. Levi, A. Nuijens, M. X. Sliwkowski, and R. P. Bunge.** 1995. Axon-induced mitogenesis of human Schwann cells involves heregulin and p185-erbB2. Proc. Natl. Acad. Sci. USA **92:**1431–1435.
22. **Pages, G., P. Lenormand, G. L'Allemain, J.-C. Chambard, S. Meloche, and J. Pouyssegur.** 1993. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc. Natl. Acad. Sci. USA **90:**8319–8323.
23. **Raff, M. C., E. Abney, J. P. Brockes, and A. Hornby-Smith.** 1978. Schwann cell growth factors. Cell **15:**813–822.
24. **Ratner, N., R. P. Bunge, and L. Glaser.** 1985. A neuronal cell surface heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. J. Cell Biol. **101:**744–754.
25. **Reise, D. J., II, T. M. van Raaij, G. D. Plowman, G. C. Andrews, and D. F. Stern.** 1995. The cellular response to neuregulins is governed by complex interactions of the erbB receptor family. Mol. Cell. Biol. **15:**5770–5776.
26. **Salzer, J. L., and R. P. Bunge.** 1980. Studies of Schwann cell proliferation. I. An analysis in tissue culture of proliferation during development, Wallerian degeneration, and direct injury. J. Cell Biol. **84:**739–752.
27. **Salzer, J. L., R. P. Bunge, and L. Glaser.** 1980. Studies of Schwann cell proliferation. III. Evidence for the surface localization of the neurite mitogen. J. Cell Biol. **84:**767–778.

27a. **Schroering, A., and D. J. Carey.** Unpublished data.

28. **Sherr, C. J.** 1996. Cancer cell cycles. Science **274:**1672–1677.
29. **Syroid, D. E., P. R. Maycox, P. G. Burrola, N. Liu, D. Wen, K.-F. Lee, G. Lemke, and T. J. Kilpatrick.** 1996. Cell death in the Schwann cell lineage and its regulation by neuregulin. Proc. Natl. Acad. Sci. USA **93:**9229–9234.
30. **Tsakiridis, T., C. Taha, S. Grinstein, and A. Klep.** 1996. Insulin activates a p21-activated kinase in muscle cells via phosphatidylinositol 3-kinase. J. Biol. Chem. **271:**19664–19667.
31. **Vaillancourt, R. R., A. M. Gardner, and G. L. Johnson.** 1994. B-Raf-dependent regulation of the MEK-1/mitogen-activated protein kinase pathway in PC12 cells and regulation by cyclic AMP. Mol. Cell. Biol. **14:**6522–6530.
32. **Vartanian, T., A. Goodearl, A. Viehover, and G. Fischbach.** 1997. Axonal neuregulin signals cells of the oligodendrocyte lineage through activation of

HER4 and Schwann cells through HER2 and HER3. J. Cell Biol. **137:**211–220.
33. **Weinberg, R. A.** 1995. The retinoblastoma protein and cell cycle control. Cell **81:**323–330.
34. **Weinmaster, G., and G. Lemke.** 1990. Cell specific cAMP-mediated induction of the PDGF receptor. EMBO J. **9:**915–920.
35. **Wood, P., and R. P. Bunge.** 1975. Evidence that sensory axons are mitogenic for Schwann cells. Nature (London) **256:**662–664.
36. **Xiong, Y., D. D. Ginty, and M. E. Greenberg.** 1996. Coupling of the RAS-MAPK pathway to gene activation by RKS2, a growth factor-regulated CREB kinase. Science **273:**959–963.
37. **Yang, M., Y. Hosakawa, S. Kaneko, M. Tanaka, and K. Nakashima.** 1996. Structure and characterization of rat cyclin D3 promoter. Gene **181:**153–159.
38. **Zhang, S., J. Han, M. A. Sells, J. Chernoff, U. G. Knauss, R. J. Ulevitch, and G. M. Bokoch.** 1995. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J. Biol. Chem. **270:** 23932–23936.