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The Ets Domain Transcription Factor Erm Distinguishes Rat Satellite Glia from Schwann Cells and Is Regulated in Satellite Cells by Neuregulin Signaling

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Distinct glial cell types of the vertebrate peripheral nervous system (PNS) are derived from the neural crest. Here we show that the expression of the Ets domain transcription factor Erm distinguishes satellite glia from Schwann cells beginning early in rat PNS development. In developing dorsal root ganglia (DRG), Erm is present both in presumptive satellite glia and in neurons. In contrast, Erm is not detectable at any developmental stage in Schwann cells in peripheral nerves. In addition, Erm is downregulated in DRG-derived glia adopting Schwann cell traits in culture. Thus, Erm is the first described transcription factor expressed in satellite glia but not in Schwann cells. In culture, the Neuregulin1 (NRG1) isoform GGF2 maintains Erm expression in presumptive satellite cells and reinduces Erm expression in DRG-derived glia but not in Schwann cells from sciatic nerve. These data demonstrate that there are intrinsic differences between these glial subtypes in their response to NRG1 signaling. In neural crest cultures, Erm-positive progenitor cells give rise to two distinct glial subtypes: Erm-positive, Oct-6-negative satellite glia in response to GGF2, and Erm-negative, Oct-6-positive Schwann cells in the presence of serum and the adenylate cyclase activator forskolin. Thus, Erm-positive neural crest-derived progenitor cells and presumptive satellite glia are able to acquire Schwann cell features. Given the *in vivo* expression of Erm in peripheral ganglia, we suggest that ganglionic Erm-positive cells may be precursors of Schwann cells.

Key Words: **Erm; Ets domain transcription factor; Oct-6/SCIP; neuregulin; satellite glia; Schwann cells; PNS; development.**

INTRODUCTION

Glial cells of the vertebrate PNS consist of myelinating and nonmyelinating Schwann cells of peripheral nerves, the satellite glia of sensory, sympathetic and parasympathetic ganglia, and the enteric glia. All of these cell types are derived from the neural crest, but it is still unclear how their phenotypic characteristics arise (Le Douarin *et al.*, 1991; Le Douarin, 1982). In avian embryos, the Schwann cell myelin protein (SMP) is expressed only by Schwann cells but not by satellite cells or enteric glia (Dulac *et al.*, 1988). Satellite and enteric glia have the potential to express SMP when withdrawn from their ganglionic or enteric environment, suggesting that inhibitory cues suppress a glial default pathway characterized by SMP expression (Cameron-Curry *et al.*, 1993; Dulac and Le Douarin, 1991). Similarly, in mouse embryos, Schwann cells but not satellite glia express the transcription factor Krox20 (Topilko *et al.*, 1994). The microenvironment of sensory ganglia appears to negatively regulate Krox20 expression since cultured satellite cells are able to upregulate Krox20 (Murphy *et al.*, 1996). The conversion of satellite cells, as defined by the absence of Schwann cell markers, to SMP-positive or Krox20-positive Schwann cells indicates that peripheral glia are derived from a common precursor (Cameron-Curry

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et al., 1993; Le Douarin *et al.*, 1991; Murphy *et al.*, 1996). This hypothesis was supported by clonal analysis of neural crest cultures, in which bipotent precursors giving rise to both SMP-positive and SMP-negative glial cells were found (Dupin *et al.*, 1990).

The nature of the signals that regulate subtype-specific glial markers is poorly understood, but both inhibitory and inducing factors may be involved. The neurotrophins BDNF and NGF exert a positive effect on SMP expression in glial cells derived from quail dorsal root ganglia (DRG), while NT3 prevents SMP expression (Pruginin-Bluger *et al.*, 1997). A crucial role at several stages of gliogenesis has been attributed to Neuregulin1 (NRG1). NRG1, a member of the epidermal growth factor superfamily, influences growth and differentiation of various cell types including neurons and glia (Burden and Yarden, 1997; Gassmann and Lemke, 1997). In peripheral gliogenesis, the NRG1 isoform GGF2 has been shown to instructively bias multipotent neural crest stem cells and neural crest derivatives to choose a glial fate (Hagedorn *et al.*, 1999; Morrison *et al.*, 1999; Shah *et al.*, 1994), although it was not determined which glial subtypes are produced from these cultures due to the lack of appropriate markers. In the Schwann cell lineage, NRG1 promotes the survival and maturation of Schwann cell precursors (Dong *et al.*, 1995) and exerts a mitogenic and antiapoptotic effect on postnatal Schwann cells (Goodearl *et al.*, 1993; Grinspan *et al.*, 1996; Levi *et al.*, 1995; Marchionni *et al.*, 1993; Syroid *et al.*, 1996; Trachtenberg and Thompson, 1996). Mice with targeted mutations of NRG1, or of ErbB receptors transmitting NRG1 signaling, exhibit severely reduced numbers of both Schwann cell precursors in peripheral nerves and neuronal and glial cells in cranial and sympathetic ganglia (Britsch *et al.*, 1998; Erickson *et al.*, 1997; Gassmann *et al.*, 1995; Kramer *et al.*, 1996; Lee *et al.*, 1995; Meyer and Birchmeier, 1995; Meyer *et al.*, 1997; Morris *et al.*, 1999; Riethmacher *et al.*, 1997). However, the generation of presumptive satellite cells in DRG is apparently not affected in these mutants (Morris *et al.*, 1999; Riethmacher *et al.*, 1997; Woldeyesus *et al.*, 1999). The analysis of ErbB2-null embryos, in which glial cells appear to accumulate in the nerve roots of the DRG and Schwann cells are not found in peripheral nerves, led to the proposal that in wild-type animals, satellite glia emigrate from the ganglia into peripheral nerves where they acquire a Schwann cell fate (Morris *et al.*, 1999). According to this model, NRG1 signaling might be required to promote a glial differentiation state compatible with migration or might stimulate migration directly.

An early marker expressed by developing satellite glia but not by Schwann cells would greatly facilitate investigations aimed at understanding the relationship between these neural crest-derived glial subtypes. Here we show that the Ets domain transcription factor Erm (Monté *et al.*, 1994) is expressed by sensory neurons and satellite glia early in development but is not expressed at any time by Schwann cells. The NRG1 isoform GGF2 instructs Erm-positive progenitors in neural crest cultures to give rise to Erm-positive glia and regulates Erm expression in cultured satellite cells. Moreover, Erm-positive cells derived either from cultures of neural crest or from DRG are able to generate Schwann cells. Finally, while satellite glia are able to undergo a transition to Schwann cells, GGF2 does not convert Schwann cells to Erm-positive satellite glia at any stage investigated.

MATERIALS AND METHODS

Nonradioactive in Situ Hybridization Using Chromogens

Nonradioactive *in situ* hybridization with digoxigenin-labeled riboprobes was performed on frozen sections of paraformaldehyde-fixed mouse embryos (Birren *et al.*, 1993; Sommer *et al.*, 1996) with the modifications described in Paratore *et al.* (1999). NBT/BCIP were used as chromogens to visualize the hybridization signals. Antisense riboprobes were as follows: partial mouse Erm cDNA was a gift from Amgen; and murine Ets-1 was cloned by RT-PCR from 4-week mouse brain cDNA using the gene-specific primers Ets-5 (5′primer, cgggatccATGAAGGCGGCCGTCGATC) containing a *Bam*HI site (small letters) and Ets-6 (3′primer, acggtaccGGTGTATCCCAGCAGGCT) containing a *Kpn*I site. The cDNA was subjected to PCR (40 cycles: 1 min 94°C, 1 min 55°C, 1 min 72°C). The PCR products were digested with the appropriate restriction enzymes and cloned into the Bluescript (pBS) SK- vector (Stratagene Cloning Systems).

Fluorescence in Situ Hybridization Combined with Immunohistochemistry

Tyramide signal amplification (TSA) was applied to detect Dig-labeled Erm riboprobe by fluorescence *in situ* hybridization. A detailed protocol has been published elsewhere (Paratore *et al.*, 1999). Briefly, subsequent to the riboprobe hybridization, sections were treated for 15 min with freshly prepared 1% H_2O_2 in methanol in order to block endogenous peroxidase activity and washed three times with TNT (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) for 5 min each. A blocking step with TNB (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.5% blocking reagent; blocking reagent supplied by NEN Life Science Products) for 30 min at RT was followed by a 30-min incubation with HRP-coupled sheep anti-Dig antibody (Roche Diagnostics) diluted at 1:100 in TNB. Subsequently, the sections were washed in TNT and mRNA expression was detected with Cy3-labeled tyramide prepared according to the manufacturer's instructions (NEN Life Science Products). Incubation with Cy3-labeled tyramide was for 12 min.

To combine detection of mRNA with the analysis of protein expression, immunofluorescence was carried out subsequent to the last washing step of the TSA *in situ* hybridization protocol. The sections were blocked for 30 min in 10% normal goat serum, 0.3% Triton-X 100, 0.1% BSA in PBS and then incubated for 2 h with a mouse monoclonal anti-neurofilament (NF160) antibody (1:100 dilution; Sigma) or a rabbit polyclonal anti-ErbB3 antibody (1:100 dilution; Santa Cruz Biotechnology), respectively. Detection was by FITC-conjugated secondary antibodies (anti-mouse antibody, Vector Laboratories; anti-rabbit antibody, Jackson ImmunoResearch Laboratories). After being washed in PBS, the sections were mounted in AF1 (Citifluor). Samples were analyzed by confocal microscopy.

Cell Culture

Time-mated OFA rats were obtained from Biological Research Laboratories (Fullinsdorf, Switzerland). DRG were dissected from E14 or E16 embryos and dissociated by incubation in 0.25% trypsin (Gibco BRL), 0.3 mg/ml collagenase type I (Worthington Biochemical) in Ca^{2+}/Mg^{2+}-free Hank's; balanced salt solution (Amimed) for 25 min, followed by addition of 1/10 vol of FBS. The cells were centrifuged for 3 min at 1800 rpm, washed once in standard culture medium (Stemple and Anderson, 1992), and plated at approximately 300 cells per 35-mm culture dish (Corning) coated with 0.5 mg/ml poly-D-lysine (Roche Diagnostics) and 0.25 mg/ml fibronectin (FN) (Roche Diagnostics). DRG cultures were maintained in standard culture medium. In some experiments, 1 nM rhGGF2, a soluble NRG1 isoform (a gift from M. Marchionni, Cambridge NeuroScience), was added 3 h after plating the cells; alternatively, cells were treated with standard culture medium supplemented with 10% FBS and 5 μM forskolin (Sommer *et al.*, 1995; Stemple and Anderson, 1992). Neural crest cell cultures were performed as reported in Hagedorn *et al.* (1999). Sciatic nerves from E14, E16, or E18 embryos were dissected and dissociated as in Morrison *et al.* (1999). Cells isolated from sciatic nerves were then centrifuged, washed, and plated as described above for DRG cultures.

Immunocytochemistry

Living cells were labeled for the cell surface antigen p75 for 30 min in standard culture medium using a monoclonal mouse antibody (Ig192) (Roche Diagnostics) visualized by Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). To label intracellular antigens, cells were fixed in PBS containing 3.7% formaldehyde for 10 min at room temperature (RT) and permeabilized for 15 min at RT using 10% goat serum, 0.3% Triton X-100, 0.1% BSA in PBS. Rabbit polyclonal anti-Erm antibodies (Janknecht *et al.*, 1996) were affinity purified using Reacti-Gel HW-65 according to the manufacturer's instructions (Pierce). Erm labeling (antibody dilution 1:200) was performed for 2 h at RT, followed by incubation with biotin-SP-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories). Staining was visualized by the ABComplex kit (DAKO) with horseradish peroxidase development using diaminobenzidine as substrate. Rabbit anti-Oct-6/SCIP antibody (Zwart *et al.*, 1996) (a gift from D. Meijer, Erasmus University, Rotterdam, The Netherlands) was used at a 1:200 dilution in 10% goat serum, 1% Triton X-100, 0.1% BSA in PBS for 45 min at RT and visualized with Cy3-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories). To reveal glial fibrillary acidic protein (GFAP) expression, a mixture of monoclonal anti-GFAP antibodies Ab-1 and Ab-2 (IgG) (NeoMarkers; each used at a 1:100 dilution) was applied for 1 h at RT, followed by staining with either Cy3-conjugated (Jackson ImmunoResearch Laboratories) or FITC-conjugated (Vector Laboratories) goat anti-mouse IgG. Staining with monoclonal anti-NF160 antibody NN18 (IgG) (1/100 dilution; Sigma) was performed for 1 h at RT and visualized by Cy3 fluorescence.

RESULTS

Spatiotemporal Expression of Erm in Neural Crest Derivatives

In order to address whether Erm might be involved in the development of the PNS, we investigated the expression pattern of Erm in a series of *in situ* hybridization experiments focusing on the neural crest and its derivatives at various developmental stages. At early stages (E8.5), Erm is not expressed in migrating neural crest cells (Fig.1, open arrow in B) which are positive for the Ets domain transcription factor Ets-1 (arrow in Fig. 1A) (Fafeur *et al.*, 1997; Maroulakou *et al.*, 1994). As soon as neural crest cells aggregate in the presumptive DRG, Ets-1 expression is downregulated (data not shown). In E11 embryos, a small population of cells at the ventrolateral margin of the forming DRG starts to display Erm expression (arrow in Fig. 1C); by E12, Erm mRNA is abundant (Fig. 1D). Erm expression persists in the DRG up to postnatal day (P) 4, the latest time point examined (Fig. 1E). However, Erm mRNA was not detectable in presumptive Schwann cells found along peripheral nerves at any developmental stage analyzed (E9 to P4) (open arrow in Figs. 1D and 1E). Apart from its expression in DRG, Erm mRNA was also present in other peripheral ganglia, such as in developing sympathetic and cranial sensory ganglia (data not shown).

Erm Is Expressed in Neurons and Satellite Glia of Developing DRG but Not in Presumptive Schwann Cells

The *in situ* hybridization analysis did not settle the question whether Erm is expressed in neurons and/or presumptive glia within the developing DRG. Unfortunately, the available antibodies to Erm (see below) were not sensitive enough to be suitable for immunohistochemistry. Therefore, we applied a fluorescence hybridization method that we have recently established, to address the cellular expression of Erm in the developing PNS *in situ* (Paratore *et al.*, 1999). The procedure is based on TSA using fluorophore-labeled tyramide. When combined with confocal microscopy, this allows the simultaneous detection of distinct mRNA species or of mRNAs together with proteins on the cellular level. In the PNS, the NRG1 receptor ErbB3 is expressed in migrating neural crest and in developing glial cells (Meyer and Birchmeier, 1995). In agreement with previous studies (Meyer *et al.*, 1997; Morris *et al.*, 1999), prominent ErbB3-positive cells were found at the ventrolateral margins of the DRG at E12 (Fig. 2A). Hybridization of E12 mouse embryo sections with an antisense Erm riboprobe followed by ErbB3 immunofluorescence demonstrated that the ErbB3-positive presumptive glial cells of DRG coexpressed Erm mRNA (Figs. 2A–2C). A sense Erm probe did not produce fluorescent signals (data not shown). In contrast, ErbB3-positive glial precursor cells along peripheral nerves did not express Erm mRNA (Figs. 2D–2F), corroborating the conventional *in situ* hybridization experiments (Fig. 1). Thus, Erm is expressed in presumptive satellite glia but not in developing Schwann cells. In the DRG, however, Erm transcripts are not restricted to the glial lineage but are also found in neuronal cells positive for neurofilament (NF160) protein (Figs. 2G–2I). Fluorescence

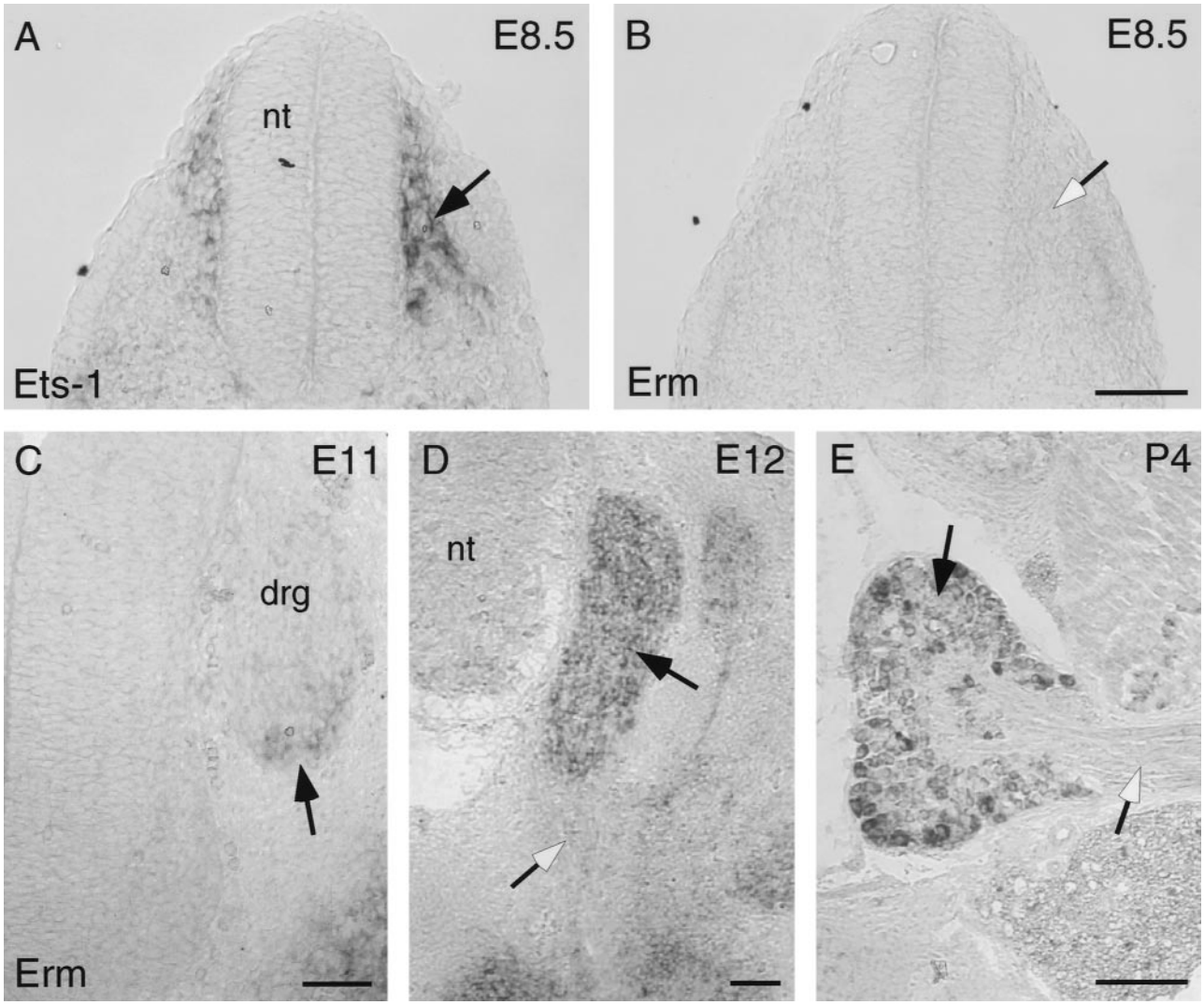


FIG. 1. Erm expression in developing dorsal root ganglia. (A, B) Erm is not detectable in migrating neural crest cells. Adjacent transverse sections through an E8.5 mouse embryo were hybridized with Ets-1 and Erm riboprobes. Migrating neural crest cells arising from the dorsal part of the neural tube (nt) are positive for Ets-1 (arrow in A) but not for Erm (open arrow in B). Transverse sections of E11 (C), E12 (D), and P4 (E) mouse trunk regions hybridized with Erm riboprobe. A few cells within the dorsal root ganglia (drg) start to express Erm at E11 (arrow in C). At E12, transcripts encoding Erm are strongly expressed in the dorsal root ganglia (arrow in D) and persist to postnatal stages (arrow in E). Note that Erm is not expressed in presumptive Schwann cells along peripheral nerves (open arrows in D and E). Bars: (A, B) 50 μm; (C, D) 100 μm; (E) 1 mm.

in situ hybridization on motor neurons did not reveal Erm expression (data not shown).

To investigate the cellular expression of Erm protein in DRG, an antibody directed to a peptide corresponding to the N-terminus of Erm (Janknecht *et al.*, 1996) was affinity purified. In transfection experiments, the antibody did not crossreact with Er81 and Pea3, the most closely related Ets domain transcription factors (data not shown) (Brown and McKnight, 1992; de Launoit *et al.*, 1997; Xin *et al.*, 1992). DRG dissected from rat E16 embryos were dissociated and fixed 4 h after plating. In such cultures, Erm was detectable by a biotin/avidin-based immunoreaction both in virtually all NF160-positive neurons and in satellite glia identified by the expression of GFAP (Figs. 3A and 3C), consistent with the fluorescence *in situ* hybridization results. Strikingly, virtually all GFAP-positive cells expressed Erm suggesting that this Ets domain transcription factor is a marker for the vast majority of developing glia in E16 DRG. In contrast, the POU transcription factor Oct-6 (also called SCIP or Tst-1; He *et al.*, 1989; Meijer *et al.*, 1990; Monuki *et al.*,

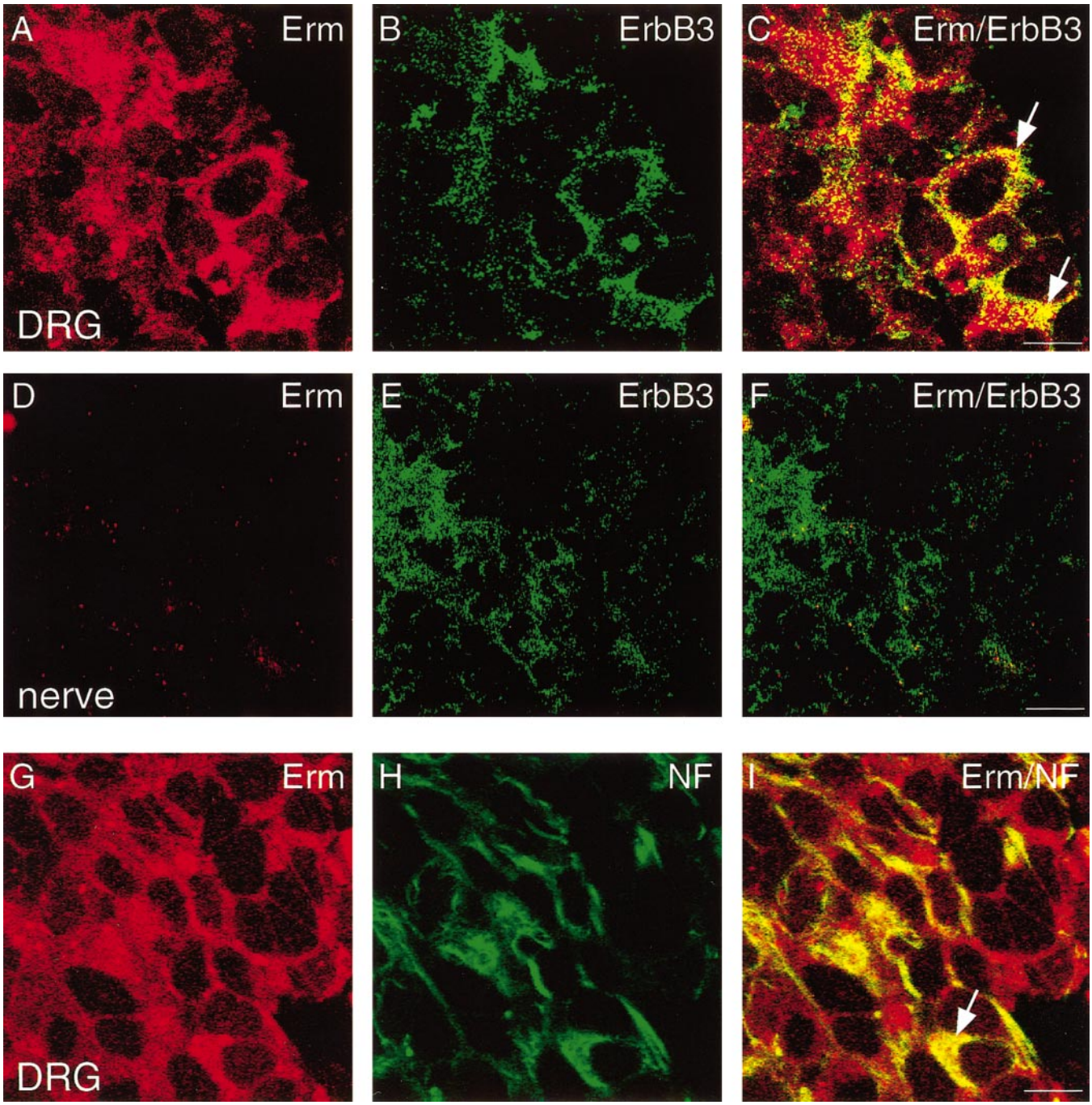


FIG. 2. Cellular localization of Erm mRNA in ErbB3-positive presumptive satellite glia and in NF160-positive sensory neurons. Sections through mouse E12 DRG (A–C, G–I) and peripheral nerve (D–F) were hybridized with Erm riboprobe (A, D, G). Fluorescence *in situ* hybridization using Cy3-coupled tyramide was followed by immunohistochemistry using anti-ErbB3 antibody (B, E) or anti-neurofilament (NF)160 antibody (H). Immunoreactions were visualized by FITC-conjugated secondary antibodies and the stainings were analyzed by confocal microscopy. Single confocal planes are shown. (C), (F) and (I) represent the confocal overlays of (A, B), (D, E), and (G, H), respectively. Yellow color indicates double-positive cells (arrows in C) revealing individual presumptive satellite glia coexpressing Erm and ErbB3. Likewise, NF-positive neurons in the DRG are double-positive for Erm mRNA expression (arrow in I). However, Erm signals were not detectable in ErbB3-positive presumptive Schwann cells in peripheral nerves (D–F). Note that at early stages in development, neuronal and glial cell types can barely be distinguished on ganglionic sections solely based on their morphology (Hall and Landis, 1992). Bars: (A–C, G–I) 10 μm; (D–F) 20 μm.

1989), which is expressed in promyelinating Schwann cells found along peripheral nerves (Arroyo *et al.*, 1998; Blanchard *et al.*, 1996; Scherer *et al.*, 1994; Zorick *et al.*, 1996), was barely detectable in satellite glia (Figs. 3B and 3D).

Differential Regulation of Erm and Oct-6 in Peripheral Glia: Role of the NRG1 Isoform GGF2 in Maintenance of Erm Expression

In cultures of DRG, satellite cells can acquire characteristics of Schwann cells. Krox20 and SMP are expressed by developing Schwann cells but not by satellite cells (Dulac *et al.*, 1988; Topilko *et al.*, 1994). Both Krox20 and SMP are upregulated in glia of serum-treated DRG cultures, revealing that at least *in vitro*, a transition from satellite cells to Schwann cells is possible (Cameron-Curry *et al.*, 1993; Murphy *et al.*, 1996). To investigate the regulation of Erm protein expression in glia acquiring Schwann cell features, cultures of dissociated DRG (E16) were incubated for 6 days in the presence of serum and forskolin which is known to result in upregulation of myelin genes (reviewed by Mirsky and Jessen, 1996; Zorick and Lemke, 1996). In such cultures, the GFAP-positive glial cells (Figs. 4D and 4K) underwent massive proliferation, became positive for the Schwann cell marker Oct-6 (Fig. 4G), and lost Erm expression (Fig. 4A). These results strongly suggest that serum/forskolin promoted the transition of $Erm^{+}/Oct\text{-}6^{-}$ satellite glia (Fig. 3) into $Erm^{-}/Oct\text{-}6^{+}$ Schwann cells (Fig. 4).

While serum/forskolin was required to induce Oct-6 expression in DRG-derived glia, downregulation of Erm expression was serum/forskolin independent. In dissociated DRG, most glial cells lost Erm expression even when cultured without serum/forskolin (Fig. 4B). However, the neurons, identified by their morphology and by NF160 expression (inset in Fig. 4E), continued to express Erm (inset in Fig. 4B). Thus, different regulatory mechanisms govern Erm expression in neurons and glia. Since Erm expression is detectable in nonneuronal components of DRG both *in situ* and in freshly isolated cultures, we considered that glial expression is lost upon cellular dissociation due to dilution of a positively acting signal. NRG1 is a good candidate for such an activity given its coordinate expression with Erm in DRG and in other developing tissues (data not shown) and since Ets domain transcription factors have been suggested to be potential targets of NRG signaling (Galang *et al.*, 1996; Janknecht, 1996; Janknecht *et al.*, 1996; Khurana *et al.*, 1999; Marais *et al.*, 1993; O'Hagan and Hassell, 1998; O'Hagan *et al.*, 1996; Sapru *et al.*, 1998; Schaeffer *et al.*, 1998; Yang *et al.*, 1996). To test this hypothesis, we examined the effect of the NRG1 isoform GGF2 on cultures of dissociated DRG. In contrast to serum/forskolin, GGF2 maintained Erm protein expression in virtually all GFAP-positive glial cells (Figs. 4C and 4F). Based on *in vivo* expression data, the presence of Erm indicates that these DRG-derived, GGF2-treated cells are satellite glia. In agreement with this, GGF2 did not upregulate expression of the Schwann cell marker Oct-6 (Fig. 4J). Hence, the expression of transcription factors that characterize distinct glial subtypes is differentially regulated by extracellular signals: endogenous Erm protein expression is maintained by the NRG1 isoform GGF2 in satellite glia without induction of Oct-6 expression. In contrast, treatment with serum/forskolin abolishes Erm expression and upregulates the Schwann cell marker Oct-6.

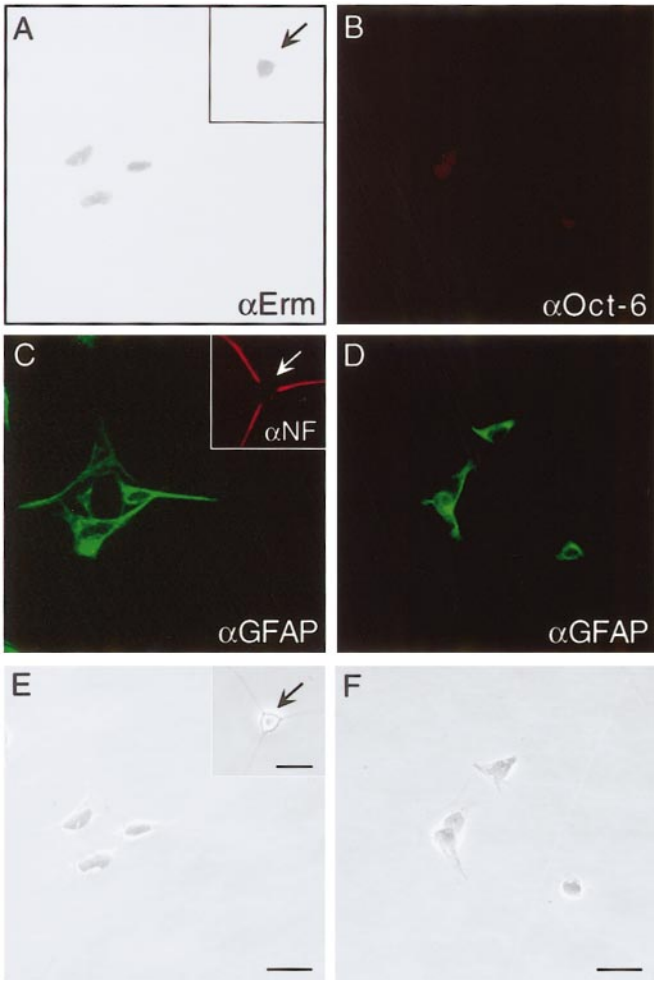
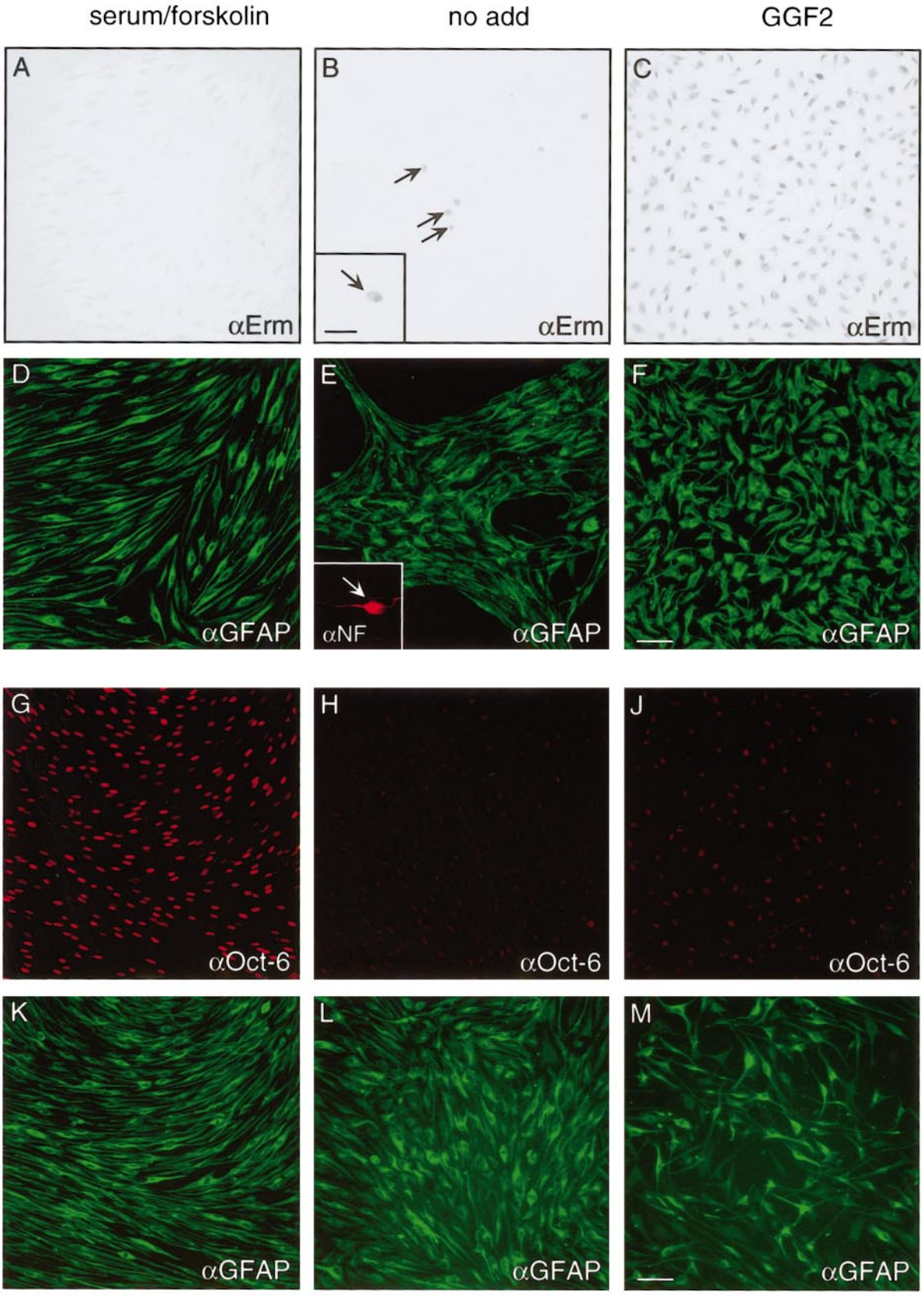


FIG. 3. Erm protein is expressed in satellite glia and in neuronal cells of E16 DRG. DRG isolated from rat E16 embryos were dissociated and the cells were fixed 4 h after plating and double labeled for Erm and GFAP (A, C, E), for Erm and NF160 (insets in A, C, E), or for Oct-6 and GFAP (B, D, F). Immunostaining for Erm (A) was visualized using a biotin/avidin-amplified horseradish peroxidase (HRP) reaction and bright-field optics. Note that Erm is expressed both in NF160-positive neurons (detected by Cy3 fluorescence; inset in C) and in GFAP-positive glia that were detected by FITC immunofluorescence. In contrast, Oct-6 (B) is not detectable in freshly isolated cells of the DRG. (E, F) Phase-contrast views. Bars, 20 μm.



Generation of Two Distinct Glial Subtypes from Erm-Positive Progenitor Cells in Neural Crest Cultures

The transition from GFAP-positive satellite glia to GFAP-positive Schwann cells might reflect a general plasticity of glial subtypes (Le Douarin *et al.*, 1991) or might point to a lineage relationship of Erm-positive cells and Schwann cells. In particular, neural crest cells might produce Erm-positive progenitor cells that generate satellite glia in the presence of GGF2 and Schwann cells in the presence of unidentified factors. Previously, cells marked by the low-affinity neurotrophin growth factor receptor (p75) have been isolated from neural crest explants and shown to be multipotent and to be instructed to a glial fate by GGF2 (Hagedorn *et al.*, 1999; Shah *et al.*, 1994; Stemple and Anderson, 1992). Our analysis of similar neural crest cultures revealed that approximately 85% of the p75-positive cells were also positive for Erm expression (Figs. 5A–5C), indicating that the early expression of Erm in neural crest cultures is independent of exogenously added GGF2. Thus, this culture system allowed us to investigate whether undifferentiated Erm-positive progenitors are able to give rise to both satellite glia and Schwann cells, independent of previous association with a DRG environment. We confirmed that addition of GGF2 to cultured neural crest cells promoted gliogenesis in the absence of neurons (Hagedorn *et al.*, 1999; Shah *et al.*, 1994) and found that the glia displayed features of satellite cells, as defined by the presence of Erm and GFAP immunoreactivity and the absence of Oct-6 staining (Figs. 5H–5L). In contrast, when Erm-positive neural crest cells were treated with serum and forskolin, neurons and glia were generated (Sommer *et al.*, 1995; Stemple and Anderson, 1992), and virtually all of the glia were Oct-6 positive but devoid of Erm expression, i.e., displaying features of Schwann cells (Figs. 5D–5G). Thus, cultured neural crest cells can give rise to two distinct glial sublineages, satellite and Schwann cells, and these Schwann cells are generated from Erm-positive progenitors.

Erm-Negative Glia Derived from DRG, but Not Developing Schwann Cells, Display Competence to Induce Erm Expression in Response to GGF2

The aforementioned experiments show that Erm-positive progenitor cells and Erm-positive satellite glia have the potential to adopt a Schwann cell fate. This could reflect sequential steps in a common lineage in which presumptive satellite cells—i.e., nonneuronal Erm-positive cells residing in the developing DRG—serve as a source for Schwann cells. Alternatively, the transition from satellite cells to Schwann cells could simply represent plasticity between peripheral glial subtypes. In the latter case, early Schwann cells would presumably also have the capacity to generate Erm-positive satellite glia. To test this hypothesis, we exposed Schwann cell precursors and early Schwann cells (Jessen *et al.*, 1994) isolated from rat E14, E16, and E18 sciatic nerves to the NRG1 isoform GGF2 and analyzed Erm expression in such cultures. In accordance with the *in situ* hybridization analysis (Figs. 1 and 2) freshly isolated developing Schwann cells did not exhibit Erm expression (data not shown). Importantly, Erm protein expression was neither induced in Schwann cell precursors nor induced in early Schwann cells even after prolonged incubation with GGF2 (Figs. 6A and 6D; shown are the data from E16 sciatic nerve cells).

A possible explanation for this result might be that GGF2 is only able to maintain Erm expression in glial cells but not to induce it. To identify whether GGF2 is able to induce Erm expression in glial cells, we made use of the DRG culture system. As described above (Fig. 4), satellite cells lose Erm expression in standard medium and were negative for both Erm (Fig. 6B) and Oct-6 (data not shown) after 3 days in culture. Upon treatment with GGF2 for an additional 3 days, however, Erm protein expression was reinduced to high levels in virtually all cells (Figs. 6C). In principle, these data could be accounted for by differential selection of residual Erm-positive cells upon GGF2 treatment of 3-day cultures rather than by induction of Erm expression in Erm-negative cells. Given that in 3-day cultures most glial cells were Erm negative (Fig. 6B), such an unexpected selective activity of GGF2 (Hagedorn *et al.*, 1999; Shah *et al.*, 1994) would have to be accompanied by substantial cell death, which we did not observe (data not shown). Hence, the NRG1 isoform GGF2 not only maintains Erm expression in satellite cells but can also reinduce it. The differential response to NRG1 signaling of glia isolated from DRG compared to developing Schwann cells prepared from peripheral nerves indicates an intrinsic difference of these glial subtypes and is consistent with an

FIG. 4. Differential regulation of Erm and Oct-6 in glial subtypes derived from DRG. Dissociated DRG were cultured for 6 days in standard medium (no add) (B, E, H, L), supplemented with fetal bovine serum and forskolin (A, D, G, K), or in the presence of the NRG1 isoform GGF2 (C, F, J, M). Cultures were double immunolabeled for GFAP (D–F, K–M) and for Erm (A–C) or Oct-6 (G–J). Cells cultured in standard medium were also double labeled for NF160 (inset in E) and Erm (inset in B). Erm staining was revealed by a biotin/avidin-amplified HRP reaction, Oct-6 and NF160 by Cy3 fluorescence, and GFAP by FITC fluorescence. In serum/forskolin, the Schwann cell marker Oct-6 was strongly expressed (G), while Erm expression (A) was not detectable in GFAP-positive glia (D). Under "no add" conditions, glial Erm expression is abolished while occasional neurons still express Erm (arrows and inset in B). Oct-6 is not expressed under these conditions (H). Upon addition of GGF2, glial cells express Erm protein at high levels (C), comparable to the Erm expression in satellite cells of freshly isolated DRG (see Fig. 3). In contrast, Oct-6 expression is barely detectable in the presence of exogenous GGF2 (J). Note that glial cells obtained in standard culture medium, in GGF2-treated cultures, or upon serum/forskolin treatment display distinct morphologies. Bars: (F, M) 50 μm; (inset) 20 μm.

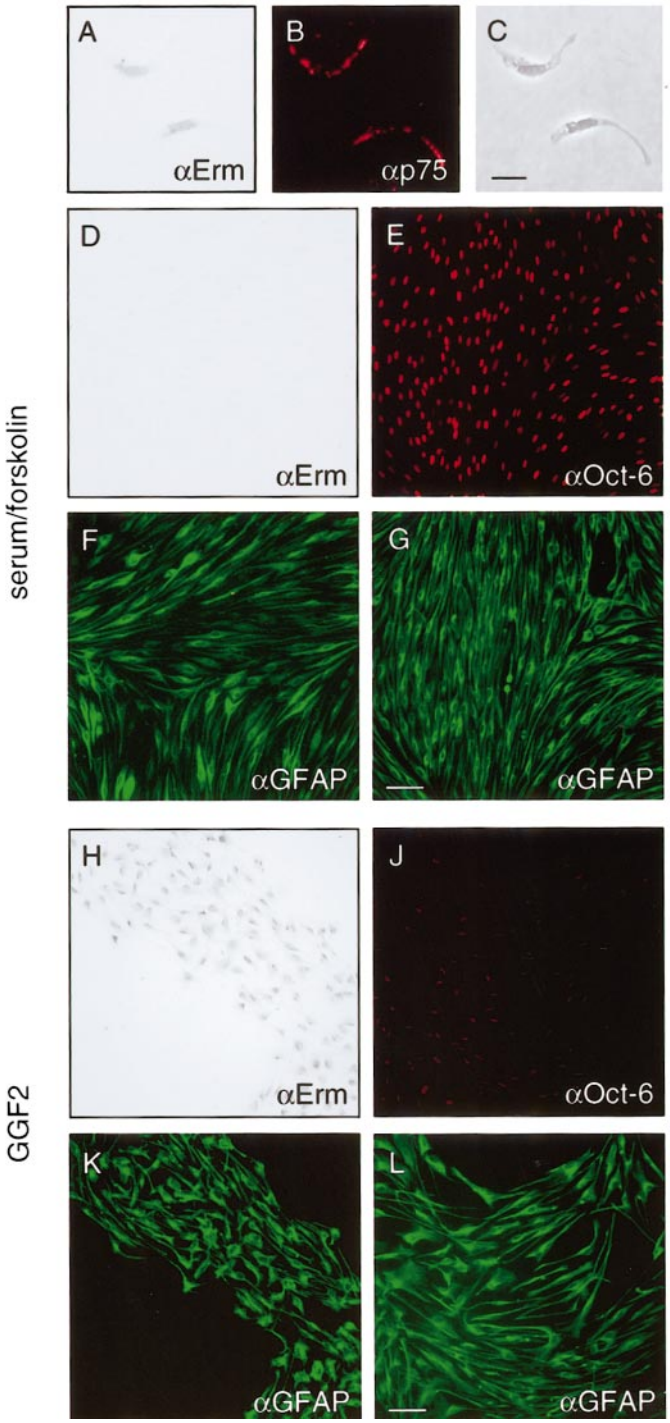


FIG. 5. Generation of distinct glial subtypes from Erm-positive progenitor cells in neural crest cultures. Neural crest cells were allowed to emigrate from rat E10.5 neural tubes in culture and were replated. In some culture dishes, cells were labeled after 3 h with anti-p75 antibody visualized by Cy3 fluorescence (B) and subsequently fixed and stained for Erm expression (A). (C) Phase-contrast image. In other culture dishes, neural crest cells were grown for 11 days in the presence of serum/forskolin (D–G) or of GGF2 (H–L) which promotes gliogenesis. These cultures were fixed and double labeled for either Erm (D, H) and GFAP (F, K) or Oct-6 (E, J) and GFAP (G, L). Erm labeling was revealed by a horseradish peroxidase reaction while GFAP was detected by a secondary antibody conjugated to FITC and Oct-6 by a fluorescent Cy3-coupled secondary antibody. Note that glial cells present in serum/forskolin-treated cultures were $Erm^{-}/Oct\text{-}6^{+}$ while glial cells generated from GGF2-treated cultures expressed Erm but not Oct-6. Bars: (C) 20 μm; (G, L) 50 μm.

irreversible developmental step from presumptive satellite cells to Schwann cells.

DISCUSSION

Identifying traits that distinguish between neural crest-derived glial subtypes are prerequisites to study their developmental relationship. We have shown here that the Ets domain transcription factor Erm is differentially expressed and regulated in distinct subsets of peripheral glia (Fig. 7). To our knowledge, Erm is the first marker shown to be expressed in presumptive satellite glia of sensory ganglia at early developmental stages but not in Schwann cells of the peripheral nerves. Maintaining Erm expression in cultured satellite cells requires the continuous presence of a positive signal that can be provided by the NRG1 isoform GGF2. GGF2 also promotes the generation of satellite cells from Erm-positive precursor cells in neural crest cultures, but does not induce the Schwann cell marker Oct-6. Serum and forskolin cause cultured Erm-positive neural crest cells and presumptive satellite glia to generate Oct-6-positive Schwann cells, while a similar transition from developing Schwann cells to satellite glia upon treatment with GGF2 was not observed. Because *in vivo* Erm-positive cells are detected in developing peripheral ganglia but not in peripheral nerves, our combined data suggest that Schwann cells are likely derived from ganglionic Erm-positive progenitors.

Regulation of the Ets Domain Transcription Factor Erm by NRG1 Signaling

The response of presumptive satellite cells to the NRG1 isoform GGF2 is reflected by regulated expression of endogenous Erm. Signaling of NRG1 is transmitted by the receptors ErbB3 and ErbB4 and their coreceptors EGFR/ErbB1 and ErbB2 (Burden and Yarden, 1997; Gassmann and Lemke, 1997; Riese and Stern, 1998). All ErbB receptor combinations activate the Ras/MAP-kinase signaling cascade (Ben-Levy *et al.*, 1994; Hynes and Stern, 1994; Kim *et al.*, 1995; Marte *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996; Si *et al.*, 1996; Tansey *et al.*, 1996). Members of the Ets domain transcription factor family are candidates for playing a role in NRG1 signaling since several vertebrate Ets domain transcription factors are transactivated by Ras/MAP-kinase

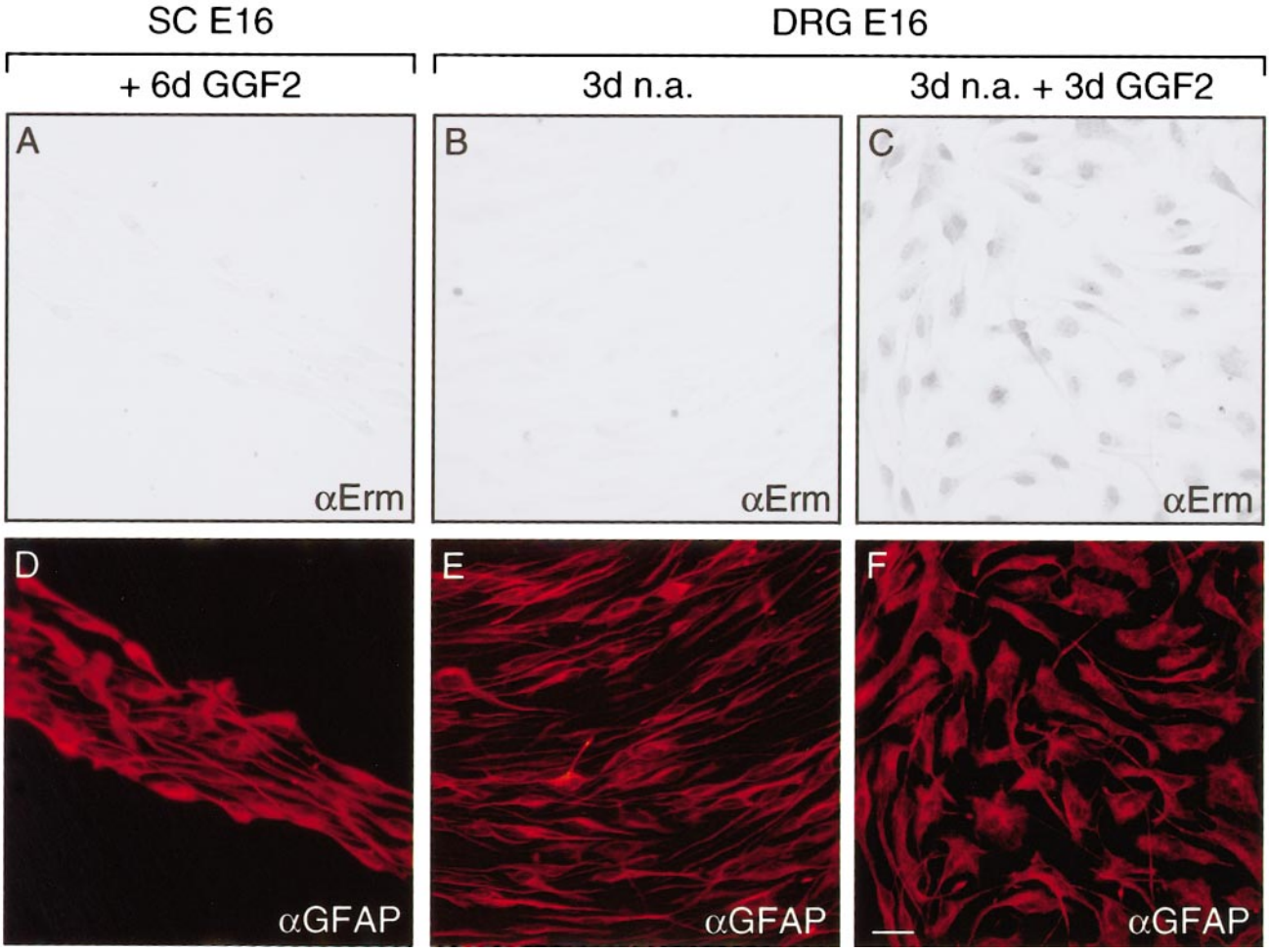


FIG. 6. The NRG1 isoform GGF2 induces Erm expression in DRG-derived glia but not in early Schwann cells. (A, D) Schwann cells were isolated from E16 sciatic nerve and cultured for 6 days (d) in the presence of GGF2. Cultures were fixed and double labeled for both Erm (A) and GFAP (D). Note that the HRP immunoreaction did not reveal Erm expression in GFAP-positive Schwann cells (visualized by Cy3 immunofluorescence). (B, C, E, F) Dissociated E16 DRG were incubated in standard medium (n.a., no add) for 3 days. While some culture dishes were fixed thereafter, sister dishes were cultured for an additional 3 days in the presence of GGF2. Double labeling for Erm (B, C) and GFAP (E, F) revealed that in standard medium Erm expression is downregulated in DRG-derived glia, but is reinduced upon treatment with GGF2. Bar, 20 μm.

activity (de Launoit *et al.*, 1997; Janknecht, 1996; Janknecht *et al.*, 1996; Karin, 1994; Marais *et al.*, 1993; O'Hagan *et al.*, 1996; Wasylyk *et al.*, 1993; Yang *et al.*, 1996). The Ets domain transcription factors Pea3 and Ets-2 can also be transactivated by overexpression of ErbB2 in a heterologous cell system (Galang *et al.*, 1996; O'Hagan and Hassell, 1998) and Pea3 was found to be upregulated concomitantly with ErbB2 in breast tumors (Benz *et al.*, 1997; Trimble *et al.*, 1993). Moreover, Ets factor-binding sites were shown to be crucial for NRG1- as well as MAP-kinase-dependent regulation of utrophin and acetylcholine receptor (AChR) δ and ϵ subunit gene expression (Khurana *et al.*, 1999; Sapru *et al.*, 1998; Schaeffer *et al.*, 1998). The effect of NRG1 signaling on AChR δ and ϵ transcription appears to be mediated via the Ets domain transcription factor GABPα (Schaeffer *et al.*, 1998). The identity of cell-endogenous Ets factors which mediate NRG1 signaling in neural development, however, has not been elucidated. Our data demonstrating regulation of Erm expression by NRG1 are consistent with the hypothesis that the Ets domain transcription factor Erm might mediate cellular responses to NRG1 signaling during development of satellite glia. This is also supported by our findings that Erm is concomitantly expressed with NRG1 at several different embryonic sites including the developing DRG (data not shown). Moreover, NRG1 signaling and Erm may share common targets. In cultured mammary tumor cells, NRG1 induces the expression of the cellular adhesion protein ICAM-1 (Bacus *et al.*, 1993) whose pro-

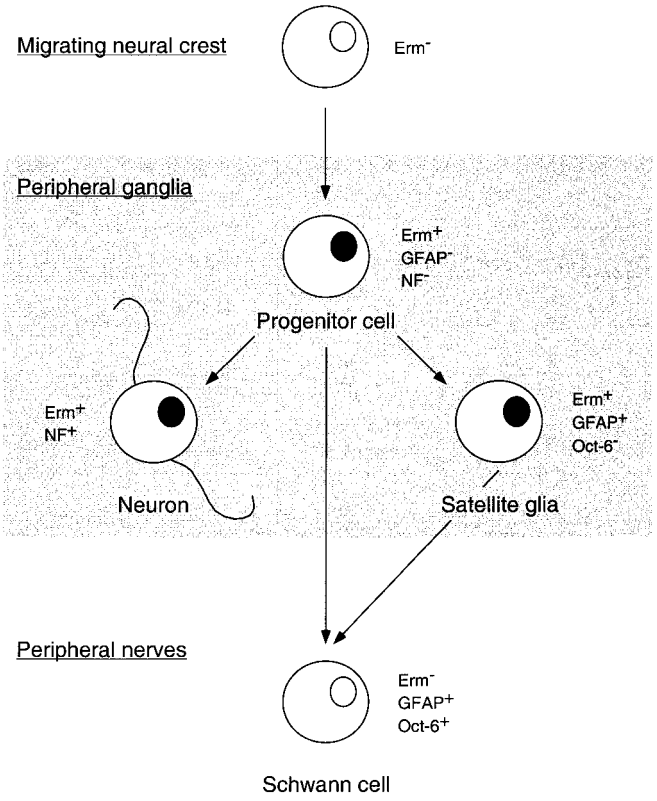


FIG. 7. Model of lineage relationship between Erm-positive cells and Schwann cells. Erm-negative migrating neural crest cells locate to the Anlage of peripheral ganglia and give rise to Erm-positive progenitor cells that are devoid of differentiation markers such as GFAP and NF. These progenitors generate Erm-positive neurons and satellite cells. Erm expression in neurons is constitutive while it is regulated in satellite glia by NRG1/GGF2. The model predicts that Erm-positive progenitors can emigrate from peripheral ganglia and have the potential to generate Erm-negative Schwann cells in peripheral nerves. This competence is maintained by GFAP-positive presumptive satellite glia. The factor(s) inducing Schwann cell traits are not yet defined but are likely to cause an irreversible progression in glia development.

moter can be activated by Erm (de Launoit *et al.*, 1998). We would expect that a direct target of NRG1 signaling should be induced in DRG-derived glia within a few hours after addition of GGF2. Reinduction of Erm in DRG cultures, however, was observed only after at least 1 day of treatment with GGF2 (data not shown), suggesting that Erm might not be an immediate early target of NRG1 signaling. Alternatively, the inhibitory mechanisms that led to Erm downregulation in DRG cultures might counteract the Erm-inducing activity of GGF2. Thus, the detailed molecular processes relating NRG1 signaling to Erm expression remain to be determined.

It is not clear whether NRG1 signaling is associated with Erm expression at all stages of neural crest development. In neural crest cultures, initial Erm protein expression appears to be independent of exogenously added NRG1. Cultured peripheral neurons constitutively express Erm irrespective of whether or not the medium contains NRG1. Moreover, *in situ* analysis of homozygous embryos (E12) containing a targeted mutation in the *ErbB3* locus (Riethmacher *et al.*, 1997) suggests that the disruption of NRG1 signaling *in vivo* does not prevent the early expression of Erm in DRG (L. Hagedorn, C. Paratore, D. Riethmacher, C. Birchmeier, U. Suter, and L. Sommer, unpublished results). However, redundancies of signaling pathways could also account for this result.

Intrinsic Differences between Peripheral Glial Subtypes

The differential response to GGF2 of DRG-derived glia compared to developing Schwann cells isolated from sciatic nerves at various developmental stages reveals intrinsic differences between presumptive satellite glia and Schwann cells. Apparently, these differences are established early in development, since neither Schwann cell precursors (E14) nor early Schwann cells (E16–E18) (Jessen *et al.*, 1994) were competent to induce Erm expression upon NRG1 treatment. Likewise, backtransplantation experiments in avian embryos indicated differences between glial cells isolated from distinct peripheral ganglia (Le Douarin *et al.*, 1991). The molecular basis for this is unclear. Although ErbB receptors are able to undergo ligand-induced combinatorial interactions with potential functional implications (Vartanian *et al.*, 1997), all neural crest-derived glia are thought to transduce NRG1 signaling via the receptor combination ErbB2/ErbB3 (Grinspan *et al.*, 1996; Levi *et al.*, 1995; Shah *et al.*, 1994; Vartanian *et al.*, 1997). Nevertheless, NRG1 signaling in developing Schwann cells and satellite glia might recruit different signal transduction molecules. As mentioned above, activated ErbB receptors stimulate the Ras/MAP-kinase signaling cascade, but additional pathways appear also to be involved (Hynes and Stern, 1994; Tansey *et al.*, 1996).

Schwann Cells Generated from Erm-Positive Progenitor Cells

Our study on Erm expression and regulation is consistent with the hypothesis (Fig. 7) that peripheral neurons, satellite glia, and Schwann cells might derive from a common Erm-positive progenitor in which Erm expression is downregulated upon acquisition of a Schwann cell fate. Since *in vivo* Erm expression first appears in developing peripheral ganglia, this model predicts that Schwann cells are generated from ganglionic progenitor cells. The differential regulation of Erm in glial subtypes raises the question whether Erm might play a role in maintaining satellite cell properties in glia of peripheral ganglia. Moreover, downregulation of Erm might be linked with competence of glial precursors to activate Schwann cell traits. In view of the proposed

lineage relationship, the activation of Schwann cell features in Erm-positive cells might be regarded as a progressive step in peripheral glial development. Accordingly, a transition from Schwann cell precursors or early Schwann cells to satellite cells would correspond to a dedifferentiation step, which is unlikely to occur and was not observed in our cultures.

Consistent with our model (Fig. 7), we have recently isolated a multipotent P0/PMP22-positive progenitor cell type from rat E14 DRG that expresses Erm but not yet the differentiation markers GFAP and NF160 (Hagedorn *et al.*, 1999; and data not shown). In culture, these cells can give rise to neurons, nonneural cells, and $GFAP^+$ glia (Hagedorn *et al.*, 1999). Depending on the extracellular factors added to the cultures, the glia generated from these progenitors were either satellite cells expressing Erm but not Oct-6 or Schwann cells expressing Oct-6 but not Erm (data not shown). Our data that Erm-positive progenitor cells derived from neural crest cultures or from E14 DRG and $GFAP^+$ satellite glia from E16 DRG display the competence to become Schwann cells corroborate the previous findings that demonstrated transitions of satellite cells to SMP-positive or Krox20-positive Schwann cells (Cameron-Curry *et al.*, 1993; Murphy *et al.*, 1996; reviewed by Le Douarin *et al.*, 1993). Moreover, the idea that Schwann cells can develop from DRG-derived nonneuronal cells has also been supported by the phenotype of mutant animals in which $ErbB2^{-/-}$ presumptive glia appear to stall in the nerve roots of DRG and, as an assumed consequence, Schwann cells in peripheral nerves are not generated (Morris *et al.*, 1999). Based on the combined data we suggest that Erm-positive presumptive satellite glia might be able to emigrate from the forming ganglia and to contribute to the Schwann cell lineage along peripheral nerves. Emigration might result in the loss of a local signal that is provided by the ganglionic environment and required to maintain Erm expression and the satellite glia state. Likely, this signal is the NRG1 isoform GGF2 that is produced by neurons (Marchionni *et al.*, 1993; Shah *et al.*, 1994) and might act in a paracrine manner to regulate Erm expression in neighboring satellite cells. Our hypothesis predicts that *in vivo* the cells emigrating from the forming ganglia encounter axons which do not yet produce local NRG1 or express NRG1 isoforms not able to maintain Erm expression. Alternatively, downregulation of Erm *in vivo* might require a repressive signal present along peripheral axons which is capable of overriding the NRG1 effect on Erm expression.

Our model does not exclude alternative cellular pathways in Schwann cell development. Clonal analysis of avian neural crest identified some founder cells which gave rise exclusively to SMP-positive Schwann cells, independent of the generation of SMP-negative satellite cells or neurons (Dupin *et al.*, 1990). However, precursor cells restricted to the Schwann cell lineage were rare in lineage-tracing studies of single premigratory and migratory neural crest cells *in vivo* (Bronner-Fraser and Fraser, 1989; Frank and Sanes, 1991; Fraser and Bronner-Fraser, 1991). Whether Erm-immunoreactive cells indeed give rise to Schwann cells *in vivo* must be addressed in future fate mapping experiments (Zinyk *et al.*, 1998).

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