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Neu Differentiation Factor Is a Neuron-Glia Signal and Regulates Survival, Proliferation, and Maturation of Rat Schwann Cell Precursors

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Summary

We show that β forms of Neu differentiation factor (NDF), homologous to acetylcholine receptor-inducing activity, glial growth factor, and heregulin, prevent apoptotic death and stimulate DNA synthesis of the E14 Schwann cell precursor, an early cell in the rat Schwann cell lineage. When precursors are exposed to NDF in defined medium, they generate Schwann cells without the requirement for DNA synthesis and with a time course that is similar to that with which Schwann cells appear in embryonic nerves in vivo. Furthermore, a neuronal signal that also mediates precursor survival and maturation is blocked by the extracellular domain of the ErbB4 NDF receptor, a protein that specifically blocks the action of NDFs. These observations provide important evidence that NDF is one of the hitherto elusive neuron-glia signaling molecules long proposed to regulate development in the Schwann cell lineage.

Introduction

Glial growth factor (GGF) has been implicated in the regulation of Schwann cell development from its first description as a mitogen for cultured rat Schwann cells (Raff et al., 1978; Marchionni et al., 1993). It has now emerged that GGF belongs to a novel growth factor group, the Neu differentiation factors (NDFs; Ben-Baruch and Yarden, 1994) that have been cloned from rat (NDF; Wen et al., 1992), human (heregulin; Holmes et al., 1992), bovine (GGF; Marchionni et al., 1993), and chick (acetylcholine receptor–inducing activity [ARIA]; Falls et al., 1993) tissues. They are encoded by a single gene but exist in an unusual variety of forms generated by alternative splicing. These forms fall into two major groups, designated α and β, according to a difference in a crucial part of the molecule, an epidermal growth factor-like (EGF-like) domain that on its own appears sufficient for receptor activation (Wen et al., 1994). The NDF receptors are two related tyrosine kinases, HER-3/ErbB3 and HER-4/ErbB4 (Tzahar et al., 1994; Wen et al., 1994). In addition, the ErbB2 protein, which is expressed in Schwann cells, probably acts as an NDF coreceptor in combination with ErbB3 and ErbB4 (Cohen et al., 1992; Peles et al., 1993; Sliwkowski et al., 1994). In various cell types, activation of these receptors promotes proliferation or differentiation (Peles et al., 1992; Falls et al., 1993; Goodearl et al., 1993; Pinkas-Kramarski et al., 1994; Vartanian et al., 1994).

NDF may also be involved in the regulation of lineage choice in the rat neural crest (Shah et al., 1994). In these experiments, crest cell clones were grown in a cocktail of known and unknown growth factors to ensure that differentiated neurons and Schwann cells were readily generated over time in culture. Addition of NDF strongly and selectively suppressed the generation of neurons in a manner consistent with the idea that NDF acted on crest cells to make the choice of the neuronal lineage less likely and that of the Schwann cell lineage more likely.

NDFs were the first polypeptide growth factors shown to control Schwann cell behavior in vitro (initially studied in this context as GGF extracted from brain), and they have remained prime candidate regulators of Schwann cell development ever since. Nevertheless, the only known effect of these proteins on cells in the Schwann cell lineage remains that originally described by Brockes et al. (1979), namely the stimulation of DNA synthesis in Schwann cells obtained from postnatal nerves.

The idea that the embryonic period of nerve development might be a time when NDF has a particularly important role to play in control of the Schwann cell lineage is supported by the cellular localization of *NDF* mRNA in rodent embryos during the second half of the gestation period (Marchionni et al., 1993; Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994). These studies have shown that the two main sources of axons in embryonic nerves, namely spinal motor neurons and dorsal root ganglion (DRG) neurons, are sites of strikingly high levels of *NDF* mRNA, whereas the glial cells within the nerve itself are negative. It is therefore pertinent to ask whether neuronally derived NDF acts to regulate glial development during this period. Analysis of glial development in the limb nerves of rat embryos (Jessen et al., 1994; Gavrilovic et al., 1995) has now made it possible to address this question in some detail.

In the rat hind limb, the embryonic period of nerve development spans about a week, from embryonic day 14 (E14), when a significant number of nerves are first present, to birth at E21. At birth, essentially all the glial cells isolated from peripheral nerves have the well-established phenotype of Schwann cells, with some cells being in the initial stage of myelination (Jessen and Mirsky, 1992). It is, however, only toward the end of the embryonic period, namely from E17 onward, that Schwann cells are present in large numbers in the major limb nerves. No such cells are present at the earliest stage of nerve development. Instead, at E14 essentially all the cells isolated from limb nerves

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are Schwann cell precursors (Jessen et al., 1994). These cells represent a distinct intermediary stage in the generation of Schwann cells from the neural crest. The precursors differ from Schwann cells in a number of ways: they die abruptly by apoptosis when removed from axonal contact in vitro; they do not express the Schwann cell marker S100 in the cytoplasm; they are not induced to synthesize DNA by fibroblast growth factor 2 (FGF2) in the presence of forskolin, a typical Schwann cell mitogen combination; and in vitro they have a flattened morphology showing many cell–cell contacts.

Thus, two separate members of the Schwann cell lineage figure in the embryonic development of major peripheral nerves: Schwann cell precursors and Schwann cells. The time between E14/15 and E17/18 is critical in nerve development, since during this period Schwann cell precursors progress to generate Schwann cells. Although this switch in glial phenotype involves change in a number of diverse cellular properties, it occurs relatively abruptly, so that only during a brief window at E16 can significant numbers of both cell types be isolated from the major limb nerves. In the present paper, we have asked whether NDF might be involved in regulation of survival, proliferation, and lineage progression of Schwann cell precursors, since these represent major issues in glial development during the embryonic period of nerve growth.

The results show that NDF blocks death and, at higher concentrations, stimulates DNA synthesis in E14 precursors. Using multiple criteria to distinguish precursors from Schwann cells, we also show that, when exposed to NDF in defined medium, precursors generate Schwann cells in vitro with a time course similar to that with which Schwann cells appear in developing peripheral nerves in vivo. DNA synthesis is not necessary for this lineage progression to occur. NDF protein is not detected in E14 precursors but is present in the cell body and axons of E14 DRG neurons. Furthermore, we demonstrate that a neuron-derived signal that mediates precursor survival and maturation can be blocked by addition of the extracellular domain of the ErbB4 NDF receptor, a protein that specifically blocks the action of NDFs. These observations provide important evidence that NDF may be one of the hitherto elusive neuron–glia signaling molecules long proposed to regulate development in the Schwann cell lineage (Bray et al., 1981; Ratner et al., 1985; Jessen et al., 1987; Bunge et al., 1990; Jessen and Mirsky, 1992).

Results

NDF Rescues Schwann Cell Precursors in a 20 hr Survival Assay

To test whether NDF could prevent the abrupt apoptotic death that precursors undergo in defined medium, precursors were exposed to various concentrations of NDFβ-2 and the corresponding α form, NDFα-2, in a 20 hr survival assay identical to that used previously (Jessen et al., 1994; Gavrilovic et al., 1995). These and all other experiments in this paper were carried out in entirely defined medium containing no serum. In these initial survival experiments,

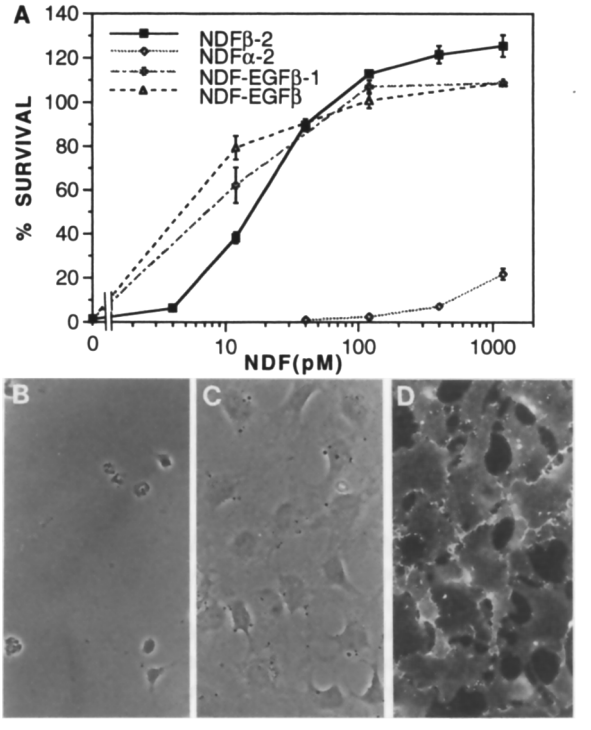

Figure 1. β Forms of NDF Prevent Death of E14 Precursors

(A) Results from a 20 hr survival assay with survival percentage calculated as described in Experimental Procedures. In this and all subsequent graphs, each point represents the average ± SEM.

(B) Representative field of E14 cultures after 20 hr in defined medium only.

(C and D) Representative field of E14 cultures after 20 hr in defined medium plus NDFβ-2 (100 pM). (D) shows L1 immunofluorescence view of (C).

Magnification, 800 ×.

the medium contained a low insulin concentration (1 nM) that is sufficient to bind to insulin receptors without significant binding to type 1 insulin-like growth factor (IGF) receptors (Sara and Hall, 1990). It was found that NDFβ-2 acted as a potent, dose-dependent suppressor of precursor death, whereas the α form, which varies in the amino acid sequence of the EGF-like domain, had very little effect (Figure 1).

Using identical conditions and assay, we investigated further which domains within the NDF molecule were responsible for the survival effect. NDFβ-1 and NDFβ-3, forms that vary in the amino acid sequence of the carboxy-terminal tail, had effects very similar to those of NDFβ-2, whereas corresponding α forms were ineffective (data not shown). Survival was also obtained with a shorter form of NDFβ-1 (residues 177–246), which lacks both the immunoglobulin-like domain and the spacer domain of NDF (NDF-EGFβ-1; Figure 1), whereas the corresponding α form was essentially ineffective (data not shown). An isolated EGF-like domain of NDFβ (residues 177–228), which also lacks the carboxy-terminal tail of NDF that in proNDF corresponds to the juxtamembrane region, was the most potent of all the forms tested (NDF-EGFβ; Figure 1).

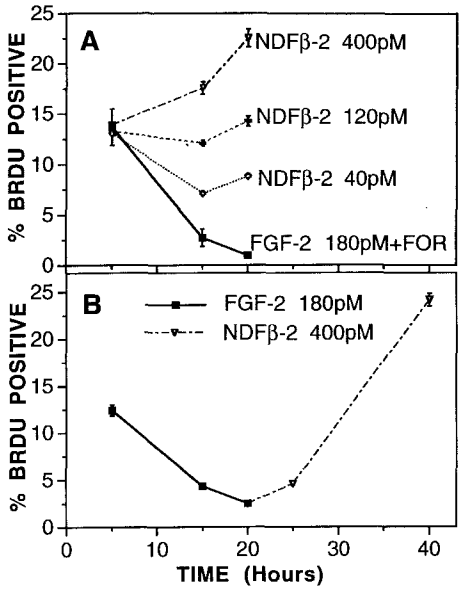

Figure 2. NDF Stimulates DNA Synthesis in E14 Precursors

The results show the percentage of L1-positive cells that were also BrdU-positive following 1.5 hr long exposures to BrdU and double immunolabeling with antibodies to L1 and BrdU. The BrdU pulses were given to sister cultures at three different times during the first 20 hr after plating. In (B) the medium was changed from one containing NDFβ-2 to one containing FGF2 plus forskolin at 20 hr, and DNA synthesis was examined using two further 1.5 hr BrdU pulses as indicated.

Thus, the EGF-like domain of NDFβs is sufficient for essentially complete suppression of death in E14 Schwann cell precursors, whereas α forms of NDF support very low levels of survival under the conditions used in these experiments.

NDF Stimulates DNA Synthesis in Schwann Cell Precursors

In the experiments described above, survival often overshot the 100 percentage mark at high NDF concentrations, suggesting that NDF could stimulate cell division.

This was tested directly by monitoring DNA synthesis during the 20 hr survival period. DNA synthesis was detected using the bromodeoxyuridine (BrdU) method and double immunolabeling with antibodies against the cell adhesion molecule L1 to identify precursors. Sister cultures were exposed to three 1.5 hr pulses of BrdU with each pulse ending at 5, 15, or 20 hr after plating. The experiment was carried out in the presence of FGF2 plus forskolin, as a nonmitogenic control (Gavrilovic et al., 1995), or various concentrations of NDFβ-2 in medium containing 1 nM insulin and 13 nM IGF1 (Figure 2A). The 5 hr pulse gave a similar proportion of BrdU-positive nuclei, irrespective of the NDF concentration used. This was expected because the time lag generally involved in mitogenic responses ensures that DNA synthesis during the first few hours in vitro reflects the previous mitogenic input seen in vivo rather than the input from growth factors present in the culture dish. Furthermore, the percentage of precursors synthesizing DNA at this point (12%–14%) was in excellent agreement with the DNA synthesis rate of these cells in vivo, measured using BrdU pulses of similar duration (Stewart et al., 1993). In the subsequent period, DNA synthesis in cells exposed to FGF2 plus forskolin rapidly decelerated, in accordance with previous findings (Gavrilovic et al., 1995). However, NDFβ-2 acted as a dose-dependent mitogen: the mitogenic effect of 40 pM was barely detectable (although survival at this NDF concentration was 100%; see Figure 3A); the initial rate of DNA synthesis was maintained in 120 pM; and it increased in 400 pM (Figure 2A).

To confirm that NDFβ-2 could also induce DNA synthesis in cells that had fallen out of division, precursors were maintained in FGF2 for 20 hr and then changed to a medium containing 400 pM NDFβ-2 (Figure 2B). This caused a rapid stimulation of DNA synthesis that, after 20 hr of exposure to NDFβ-2, was similar in magnitude to that seen 20 hr after plating using the same NDFβ-2 concentration (Figure 2A). Other forms of NDFβ, including the isolated EGF-like domain alone, also stimulated DNA synthesis and showed similar dose–response relationships (data not shown). Comparison of the doses required to support survival and stimulate DNA synthesis (Figure 2A; also see Figure 1) indicated, however, that rescue does not depend on stimulation of DNA synthesis, since low concentrations that are only weakly mitogenic nevertheless support essentially full survival. This was confirmed in subsequent experiments (see below).

NDFβs therefore act both to block death and stimulate DNA synthesis in E14 Schwann cell precursors, in contrast to FGFs, which promote survival only.

The Involvement of IGF1 in NDF-Driven Survival and DNA Synthesis

Previously, we have shown that FGF-mediated rescue of precursors depends on the presence of high concentrations of insulin or IGF1 (1 μM [5.7 μg/ml] and 6.5–13 nM [50–100 ng/ml], respectively), indicating that FGF rescue depends on activation of type 1 IGF receptors (Gavrilovic et al., 1995). The present experiments showed, however, that NDFβs could completely prevent precursor death in medium containing low insulin (1 nM) and no IGF1 (see above).

To investigate this issue further, dose–response curves for NDFβ-2-mediated survival were generated under three different conditions: in the absence of insulin and IGF1; in the presence of 1 nM insulin only, to activate insulin receptors; and in the presence of 1 nM insulin plus 13 nM IGF1, to activate type 1 IGF receptors also (Figure 3A). Even in the absence of both insulin and IGF1, NDFβ-2 was capable of complete rescue at 20 hr. In the presence of low insulin, NDFβ-2 was more potent, the ED_{50} (the concentrations giving half-maximal survival) having shifted toward lower concentration by about 5- to 6-fold. Addition of IGF1 on top of low insulin promoted survival at the lowest NDFβ-2 concentrations only, leaving the ED_{50} essentially unchanged.

Since IGF1 promoted the marginal survival generated

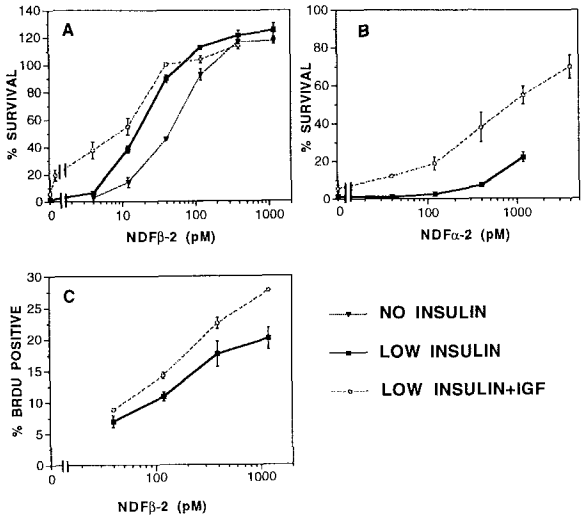

Figure 3. The Effect of Insulin Growth Factors on NDF-Mediated Survival and DNA Synthesis

(A and B) Results from a 20 hr survival assay (see legend to Figure 1). (C) NDF stimulates DNA synthesis in the absence of type 1 IGF receptor stimulation. The results show the percentage of L1-positive cells that were also BrdU-positive following exposure to various concentrations of NDFβ-2 in media with or without IGF1 as indicated.

by low NDFβ-2 doses, we tested whether IGF1 would also enhance the very low survival generated by α forms of NDF (see Figure 1). Indeed, 13 nM IGF1 markedly enhanced NDFα-2-mediated survival (Figure 3B). Under these conditions survival of about 60% could be obtained, albeit at high concentrations of NDFα-2. NDFα-1 and NDFα-3 supported survival comparable to that seen with NDFα-2 in the presence of 13 nM IGF1 (data not shown).

As a test of the involvement of type 1 IGF receptor in NDF-regulated DNA synthesis, dose–response curves for NDFβ-2-stimulated DNA synthesis were generated in the presence of 1 nM insulin only, and in the presence of 1 nM insulin plus 13 nM IGF1 (Figure 3C). DNA synthesis was monitored with the BrdU method and anti-L1 antibodies as before, except that the cells were exposed to only a single 1.5 hr BrdU pulse at 20 hr (equivalent to the last pulse in the three time point assay used previously). This showed that NDFβ-2 readily stimulated DNA synthesis in the presence of low insulin concentration (1 nM) without IGF1. Furthermore, although addition of IGF1 enhanced the mitogenic response, this effect was barely significant statistically. In one experiment, DNA synthesis in response to 400 pM NDFα-2 was measured in a similar way in the presence of 13 nM IGF1. Less than 1% of precursors were BrdU positive in this experiment.

Together, these results indicate that high concentrations of insulin or IGF1 (and therefore presumably type 1 IGF receptor stimulation) play a smaller role in NDFβ-regulated survival and DNA synthesis in Schwann cell precursors than might have been expected from previous studies on FGFs and other growth factors on cells in this lineage.

NDF, but Not FGF2, Supports Long-Term Survival of Precursors

Previously, two conditions have been shown to support the survival of Schwann cell precursors for more than 20 hr: FGF2 in the presence of high concentrations of insulin plus a low amount of serum and, alternatively, defined medium conditioned by neurons (Jessen et al., 1994; Gavrilovic et al., 1995). Both situations involve exposure to unidentified factors, and so far, defined conditions that allow the survival of cells removed from E14 nerves for longer periods have not been identified.

We therefore examined whether NDFβ-2 could support longer-term precursor survival and compared it with FGF2. Both factors were applied in defined medium containing 1 nM insulin and 13 nM IGF1. Survival was assessed as before, except that in addition to a 20 hr survival period, some coverslips were maintained for 44 and 68 hr prior to immunostaining with anti-L1 antibodies and counting (Figure 4A). In NDF, cell number at the end of the 3 day period was somewhat higher than at 3 hr, whereas in FGF2 cell number fell rapidly after the first day. Using 1.5 hr BrdU pulses at the end of the first and second day in NDFβ-2, we found BrdU incorporation in L1-positive precursors of 3.0% and 3.8%, respectively. Thus, NDFβ-2 at 32 pM supports the survival of Schwann cell precursors for several days, generating some increase in cell number toward the end of the period, owing to a low level of DNA synthesis. In FGF2, however, the cells died rapidly after the first day.

Although E14 precursors after 20 hr in FGF2 could not at this point be rescued by FGF2, we found that they could be rescued completely by NDF. This was shown in experiments in which E14 precursors were maintained in FGF2 for 20 hr and then changed to fresh FGF2 or to medium containing NDFβ-2: essentially all the cells changed from FGF2 to NDF survived the next 2 days, whereas most of the cells that remained in FGF2 medium died during the same period (Figure 4B). This shows that, after a 20 hr exposure to FGF2, precursors are not irreversibly set on a death course but have selectively lost responsiveness to FGF2.

In the Presence of NDF, Precursors Generate Schwann Cells on Schedule and in the Absence of DNA Synthesis

Having shown that NDFβ-2 supported full survival of the L1-positive (or nerve growth factor receptor [NGFR]–positive) cells, obtained as Schwann cell precursors from E14 nerves, for at least 4 days, we asked whether the developmental status of these cells changed during the culture period. If NDFβ-2, applied under the defined conditions used here, mimicked the conditions prevailing during embryonic nerve development in vivo, essentially all the precursors would convert to cells with the Schwann cell phenotype during a 4 day period (E14 + 4 = E18), since, in vivo, cells in E14/15 nerves have the precursor phenotype, whereas cells in E17/18 nerves have a Schwann cell phenotype (Jessen et al., 1994; Gavrilovic et al., 1995). Alternatively, it was possible that NDFβ-2 blocked apoptosis in E14 cells without providing the conditions required for

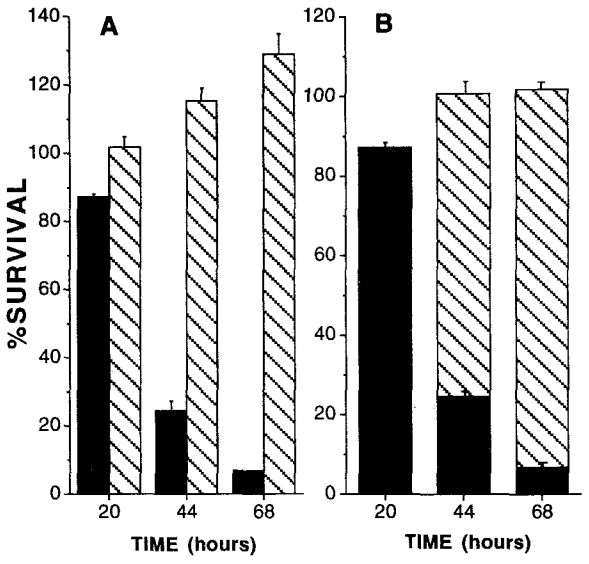

Figure 4. Long-Term Survival of E14 Precursors

(A) NDF (hatched bars) supports long-term survival, whereas the effect of FGF2 (closed bars) is transient. Survival was measured (see legend to Figure 1) in sister cultures at different times after plating and in the presence of NDF (32 pM) or FGF2 (180 pM, a supramaximal concentration in a 20 hr assay).

(B) NDF (hatched bars), but not FGF2 (closed bars), rescues precursors following a 1 day exposure to FGF2. All cultures were exposed to FGF2 (180 pM) for 1 day. Some cultures were then fixed and used for survival assessment (column at 20 hr), while other cultures were changed to fresh medium containing FGF2 or NDFβ-2 (32 pM) as indicated. The survival of these cultures was measured after a further 1 day (columns at 44 hr) or 2 days (columns at 68 hr). Survival was measured as before (see legend to Figure 1).

lineage progression and Schwann cell generation. In cultures that initially consist of precursors only, Schwann cell generation can be monitored quantitatively by examining the gradual appearance of cells that express cytoplasmic S100 and survive in defined medium in vitro when plated at moderate cell density. Furthermore, E14 precursors, unlike neonatal Schwann cells, do not synthesize DNA in response to the combination of FGF2 and forskolin (see above). This suggested an additional feature differentiating the precursor and Schwann cell phenotypes. In the following experiments, we first confirmed this by showing that a mitogenic response to FGF2 plus forskolin appears in vivo between E15 and E17. We then used the three criteria of cytoplasmic S100 expression, survival ability and mitogenic response to FGF2 and forskolin, to monitor the generation of Schwann cells from precursors in vitro.

The Developmental Appearance of a Mitogenic Response to FGF2

To determine when cells freshly isolated from embryonic nerves started to respond to FGF2 by DNA synthesis, cell cultures were prepared from E14, E15, E17, E18, and newborn nerves and maintained in FGF2 plus forskolin. During the first 20 hr after plating, DNA synthesis was measured using the three time point BrdU assay carried out previously for E14 cells only (Figure 5A). This showed that at 5 hr DNA synthesis was considerably greater in E17 and E18 cells than in E14, E15, or newborn nerves, reflecting the peak of DNA synthesis seen during normal nerve development in vivo (Stewart et al., 1993). Also, in the presence of FGF2 and forskolin, DNA synthesis decelerated rapidly in the cell population from E14 and E15 nerves. The same result was obtained when the FGF2 concentration was increased from 180 pM, which has supramaximal effects on DNA synthesis in neonatal Schwann cells, to 600 pM (data not shown). In contrast, the considerable initial synthesis level was maintained in E17 cells and elevated in E18 cells. As expected, the three time point assay also revealed a stimulation of DNA synthesis by FGF2 and forskolin in newborn cells. The simplest interpretation of these results is that FGF2 plus forskolin in the medium used here stimulates DNA synthesis in Schwann cells but not in Schwann cell precursors. The mitogenic response to these factors can therefore be used, together with previously defined criteria, to monitor differentiation in the Schwann cell lineage and to examine the generation of Schwann cells from precursors.

The Generation of Schwann Cells from E14 Precursors In Vitro

Cells were prepared from E14, E15, E17, E18, and newborn nerves and maintained in NDFβ-2 in defined medium. After 1–4 days in vitro their phenotype was assessed quantitatively. For each assessment, the cells were removed from the culture dish, replated on coverslips, and examined for ability to survive for 20 hr in defined medium only; cytoplasmic S100 immunoreactivity at 3 hr after plating; and DNA synthesis in response to FGF2 plus forskolin at 5, 15, and 20 hr after plating. The replating strategy was used to eliminate the effects of growth factors bound to the culture substrate in the period prior to assessment, and the methods used to examine precursor versus Schwann cell phenotype were identical to those used previously on cells freshly obtained from nerves at different ages.

Initially, survival was measured in cells from E14, E15, E17, and newborn nerves after 1 day in vitro (Table 1). E14 + 1 (= 15) day cells survived poorly, as did cells from E15 nerves, whereas E15 + 1 (= 16) day cells showed increased survival, in agreement with the increased survival shown by cells removed from E16 nerves when compared with cells from E15 animals. The assay registered a much better survival of newborn cells after 1 day in vitro, in agreement with the observation that these cells survive well when tested directly after removal from the nerve. E17 + 1 (= 18) day cells showed survival similar to that of E18 cells. We then measured survival of E14 cells by replating after 2, 3, and 4 days in vitro (Table 1). E14 + 2 (= 16) day cells survived significantly better than E14 + 1 (= 15) day cells. A much larger increase in survival was, however, seen between E14 + 2 (= 16) day cells and E14 + 3 (= 17) day cells, and the survival of E14 + 4 (= 18) day cells was somewhat higher than that seen with E14 + 3 day cells and comparable to that seen with newborn + 1 day cells. All of this is in line with the survival shown by cells removed from nerves at the equivalent embryonic ages.

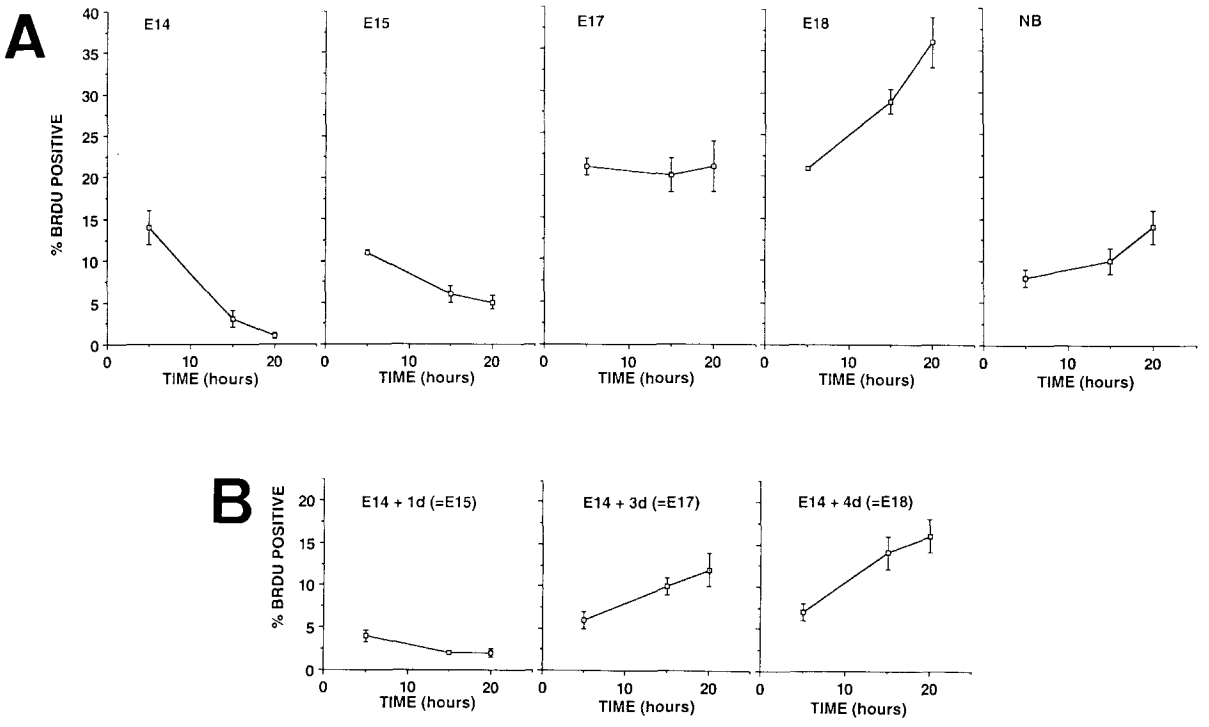

Figure 5. A Mitogenic Response to FGF2 plus Forskolin Distinguishes Schwann Cells from Precursors

(A) The results show the percentage of L1-positive cells that also have BrdU-positive nuclei following three 1.5 hr long exposures to BrdU during the first 20 hr after plating of cells removed from animals at different developmental stages as indicated. All cultures were exposed to FGF2 (180 pM) plus forskolin (5 μM).

(B) The results are from a three time point BrdU assay as described for (A). The assay was carried out during the first 20 hr after replating of cells that had been obtained from E14 animals and maintained for 1, 3, and 4 days in NDFβ-2 (40 pM).

Examination of cytoplasmic S100 immunoreactivity in this type of experiment revealed a comparable picture (Table 1). Few E14 + 1 (= 15) day cells were S100 positive, whereas about half of the E14 + 2 (= 16) and E15 + 1 (= 16) cells bound S100 antibodies. The great majority of E14 + 3 (= 17) and E14 + 4 (= 18) day cells were S100 positive. These figures are in good agreement with those obtained by immunolabeling cells from nerves at equivalent ages.

In a further test of whether E14 precursors acquired the

Table 1. Change in Survival Ability and S100 Expression during Precursor/Schwann Cell Conversion

Cells (Age When Removed from Nerve + Days In Vitro)	Percentage of Cells That Express S100 ± SEM	Survival Percentage ± SEM
E14 + 0	4 ± 3	1 ± 2
E15 + 0	4 ± 4	3 ± 2
E14 + 1	15 ± 2	12 ± 2
E16 + 0	42 ± 6	16 ± 5
E14 + 2	41 ± 4	30 ± 3
E15 + 1	47 ± 7	18 ± 3
E17 + 0	95 ± 2	87 ± 5
E14 + 3	75 ± 3	71 ± 2
E18 + 0	96 ± 2	98 ± 2
E14 + 4	84 ± 3	85 ± 9
E17 + 1	93 ± 1	92 ± 6
Newborn + 0	93 ± 6	100 ± 4
Newborn + 1		79 ± 5

Plus 0 days in vitro indicates experiments in which the survival assay was carried out immediately after removal of cells from nerves, and the survival percentages in these experiments and the percentage of cells expressing cytoplasmic S100 are obtained from Jessen et al., 1994. They are presented here to make direct comparison with the present results easy. Plus 1–4 days in vitro indicates experiments in which cells were maintained for 1–4 days in vitro prior to the survival assay (see Experimental Procedures). Each experiment was carried out using duplicate or triplicate coverslips, and each figure represents the average of three experiments, except for experiments with newborn cells, which were carried out twice. In this case, the figure represents standard deviation from a pooled total of 4 coverslips.

Schwann cell phenotype on schedule, DNA synthesis in response to FGF2 plus forskolin was examined in cells prepared from nerves at E14 and maintained in NDF for 1–4 days (see Figure 5B). The phenotype of the cells was assessed during the first 20 hr following replating, using the three time point BrdU assay described previously. FGF2 plus forskolin was not mitogenic for E14 + 1 (= 15) day cells, although this combination stimulated DNA synthesis in E14 + 3 (= 17) day cells and in E14 + 4 (= 18) day cells. In other experiments, we showed that exposure to 5 μM forskolin for 20 hr induced high levels of P_0 mRNA in precursors exposed to NDFβ-2 for 4 days and replated, as expected if these cells were Schwann cells (data not shown).

Together, these observations show that, when cultures of E14 precursors are exposed to NDFβ-2 under defined conditions, the phenotype of the cell population changes gradually but comprehensively from that characterizing precursors to that of Schwann cells. This occurs with a time course similar to that of Schwann cell appearance in nerves during development in vivo.

DNA Synthesis during Schwann Cell Generation

When DNA synthesis was measured, using 1.5 hr BrdU pulses, during the 1–4 day culture periods that preceded the phenotypic assessment described in the previous section, it was found to range between 10% and 20%, which is comparable to the levels of DNA synthesis in vivo during the period of E14–E18 (Stewart et al., 1993). We now asked whether the gradual replacement of cells with the precursor phenotype by cells with the Schwann cell phenotype in the cultures described above was dependent on this rate of cell division, or whether it could take place in cultures where DNA synthesis was insignificant.

In these experiments we took advantage of the observation that at low concentrations NDFβ-2 can support substantial survival while inducing very little DNA synthesis (see above). Cells removed from E14 nerves were therefore maintained in NDFβ-2 at 28 pM for 4 days (E14 + 4 = 18) prior to being assessed for precursor/Schwann cell phenotype on the bases of survival, S100 expression, and FGF2 responsiveness as described above. DNA synthesis measured only 1.1% ± 0.4%, 1.9% ± 0.6%, and 1.9% ± 0.8% on days 1, 2, and 3 in vitro, respectively. When the phenotype of these cells was assessed on the fourth day, 67% ± 8% of the L1-positive cells survived for 20 hr in defined medium, 89% ± 3% expressed cytoplasmic S100, and DNA synthesis in the presence of FGF2 plus forskolin increased from 3.0% to 13.2% during a 20 hr period. Thus, about a 10-fold drop in DNA synthesis to very low levels did not materially alter the extent of Schwann cell generation. To investigate further the relationship between DNA synthesis and Schwann cell generation from precursor cells, E14 precursors were maintained in NDFβ-2 at 16 pM for 3 days in the continuous presence of BrdU in two separate experiments. The cells were then assayed for precursor/Schwann cell phenotype by measuring 20 hr survival as described above, except that at the end of the 20 hr period, we carried out double immunolabeling for BrdU and L1. This showed that 58% of the surviving L1-positive cells (i.e., Schwann cells) had not incorporated BrdU during the 3 day period, whereas the relative number of cells that had acquired the Schwann cell phenotype in this low NDFβ-2 concentration was unchanged from that seen at higher concentrations.

These experiments make it very unlikely that the Schwann cells generated in these cultures arise from a hypothetical population of rapidly dividing stem cells, and they show that at least the great majority of these cells must have originated in E14 precursors that converted to Schwann cells without the requirement of cell division.

Soluble ErbB4 Protein Blocks Survival Activity in Neuron-Conditioned Medium

Previously, defined medium conditioned by purified cultures of DRG neurons was shown to contain proteinaceous survival agent(s) that suppressed apoptosis of precursors in a dose-dependent way and allowed E15 precursors to progress to Schwann cells during a period of 5 days without the requirement for cell division (Jessen et al., 1994). Since these effects are similar to those of NDF, we now asked whether this activity was NDF. This was done by testing whether the activity could be blocked by addition of a soluble hybrid protein containing the NDF binding site of the ErbB4 receptor.

To ensure unambiguously that the activity in the conditioned medium was of neuronal origin, we used DRG neurons purified by immunopanning to yield cultures in which >95% of the cells were neurons at the time of medium collection (Figure 6A, upper panel). We confirmed immunocytochemically that the neurons in these cultures expressed NDF protein (Figure 6A, lower panel). Defined medium without serum was conditioned by these cultures for 24 hr. Using the 20 hr survival assay described previously and cells from E14 nerves, this medium was then tested alone or following addition of 1 or 3 μg/ml ErbB4 protein. Control experiments involved the addition of ErbB4 to survival medium containing FGF2 together with IGF1 and forskolin to test for nonspecific effects, and the addition of ErbB4 to survival medium containing recombinant NDFβ-2 to verify that the receptor protein blocked the effects of NDF under the prevailing experimental conditions. ErbB4 had no effect on FGF-mediated survival of E14 cells but strongly inhibited the effect both of NDFβ-2 and neuron-conditioned medium (Figure 6B). In other experiments, we found that the ErbB4 protein also caused a 65% reduction of precursor survival in a 20 hr survival assay of neuron–precursor cocultures. In this system, precursors are seeded on top of extremely sparse cultures of pure neurons, and survival of precursors is mediated by contact with axons, in the absence of any detectable survival activity in the culture medium (Z. D. unpublished data).

The ErbB4 receptor has not been found to bind to factors other than those of the NDF family. It is therefore very likely that the major active ingredient in the neuron-conditioned medium is NDF. ErbB4 exerted a stronger inhibitory effect on 40 pM NDFβ-2 than it did on the neuron-conditioned medium. This could be due to the concentration of NDF in the neuron-conditioned medium being higher than 40 pM. Alternatively, the conditioned medium may contain

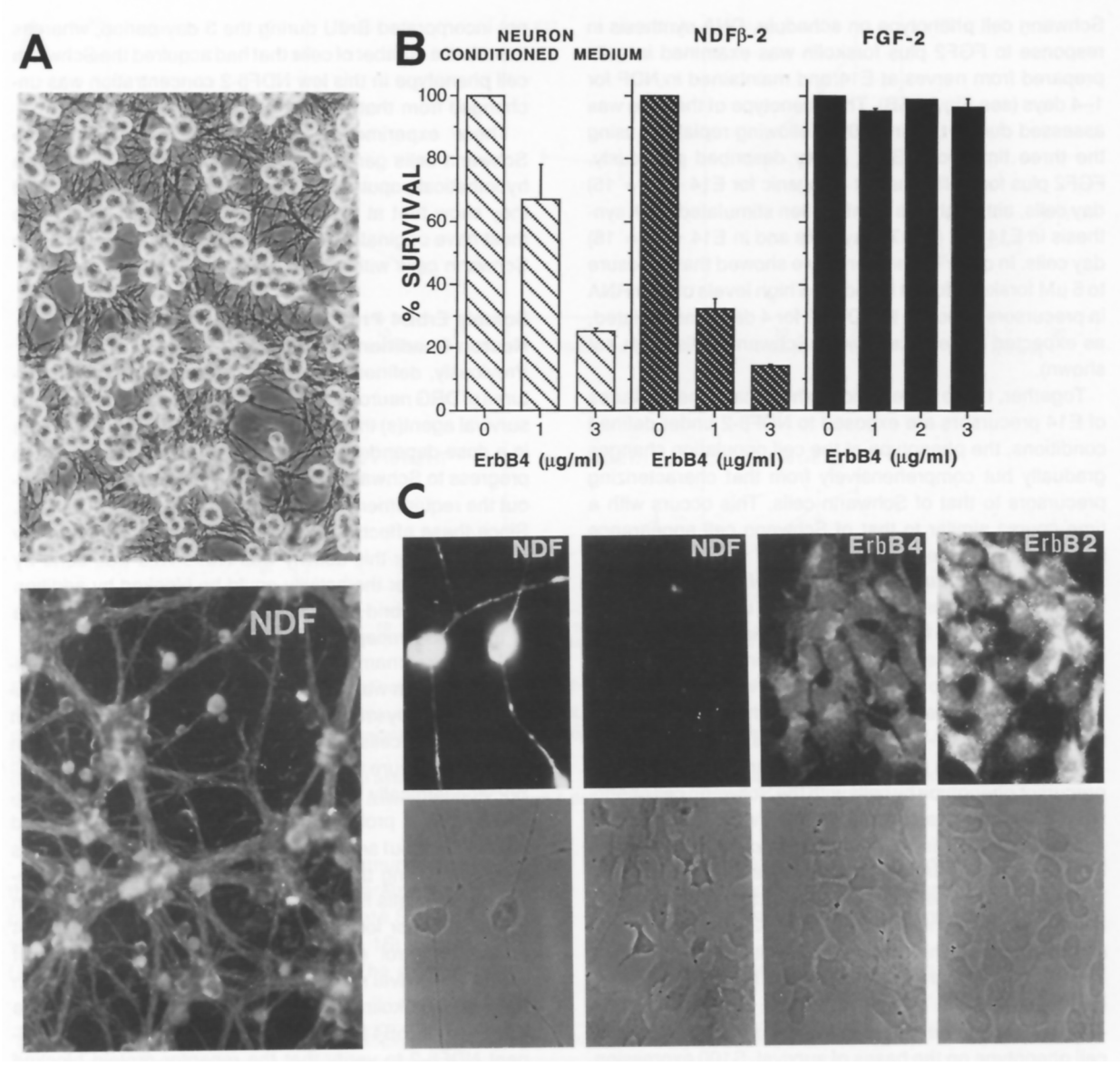

Figure 6. The Neuron-Derived Precursor Survival Signal Is Likely to be NDF

(A) The upper panel is a representative phase-contrast view showing the appearance of the highly purified, immunopanned DRG cultures used to generate conditioned medium. The photograph was taken 48 hr after plating; over 95% of the cells in these cultures were neurons (magnification, 400×). The lower panel shows NDF immunoreactivity in a DRG culture, similar to the one shown in the upper panel. The cells were labeled using the 5D6A anti-NDF antibody. Note immunolabeling of neuronal cell bodies and bundles of naked neurites (magnification, 600×).

(B) Soluble ErbB4 protein blocks the survival activity in DRG-conditioned medium and the survival effect of NDFβ-2; the survival effect of FGF2 is unaffected. The results are from 20 hr survival assays.

(C) Immunolabeling of E14 DRG neurons and precursors using antibodies against NDF, ErbB4, and ErbB2, as indicated on the immunofluorescence micrographs in the upper panels. The lower panels show the corresponding phase-contrast views. Note that both cell bodies and processes of the two DRG neurons are NDF positive (antibody 5D6A), whereas E14 precursors are NDF negative. ErbB4 immunolabeling is uniform and present in essentially all precursors, whereas ErbB2 labeling varies more from cell to cell (controls were negative; see Experimental Procedures). Magnification, 700–800×.

other neuron-derived factors that synergize with NDF to promote precursor survival.

Localization of NDF and NDF Receptors in E14/15 Cells

To localize NDF protein in E14 DRG neurons, we used anti-NDF antibodies and immunohistochemical methods. When neurons dissected from E14 animals were examined after 1–2 days in vitro, strong immunolabeling was seen in both the cell bodies and neuronal processes (Figure 6C). No significant antibody binding was detected in E14 precursors in similar immunolabeling experiments (Figure 6C). Comparable results were obtained using three different anti-NDF antibodies (see Experimental Procedures).

Expression of the ErbB4 NDF receptor and of the ErbB2

NDF coreceptor was also examined by immunohistochemistry; suitable antibodies against the rat ErbB3 receptor were not available (Figure 6C). Essentially all E14 precursors showed speckled and apparently membrane-associated immunolabeling when examined with antibodies against ErbB4. The large majority of precursors also bound antibodies against ErbB2, but the intensity of the immunolabeling was more variable in this case.

Discussion

The present results show that β forms of NDF regulate survival and DNA synthesis of E14 Schwann cell precursors. NDF also supports orderly lineage progression of these cells since, in the presence of NDF, they form Schwann cells in vitro with a time course that is similar to that of Schwann cell appearance in major embryonic nerves in vivo. Under defined conditions in media without serum, no other growth factor is presently known to exert comparable effects on these cells. A protein that regulates precursor development in a similar way was previously described in defined medium conditioned by DRG neurons (Jessen et al., 1994). Here we report that the effect of this protein is abolished by exposure to a soluble form of the extracellular domain of the ErbB4 NDF receptor, a protein that binds to NDF with a very high degree of specificity (Culouscou et al., 1993; Plowman et al., 1993a, 1993b; Tzahar et al., 1994). Together, these observations identify NDF as a neuron–glia signal that controls survival and differentiation of Schwann cell precursors in a simple and well-defined in vitro model of neuron–glia interactions during embryonic nerve development. NDF is likely to carry out a similar function in vivo, particularly in view of the strikingly high *NDF* mRNA expression in those neurons that form embryonic peripheral nerves (Marchionni et al., 1993; Orr-Urtreger et al., 1993; Meyer and Birchmeier, 1994) and in view of the present observation that these cells express NDF proteins in cell bodies and processes while Schwann cell precursors show low or no NDF expression.

Although the existence of neuronal signals responsible for driving differentiation in the Schwann cell lineage has been a central idea in studies on PNS development for a long time, these signals have remained unknown at the molecular level. The molecular identification of an important component of these signals is therefore a significant step forward.

NDF and the Suppression of Cell Death

NDF potently and completely blocked the acute apoptotic death of E14 precursors. This effect was seen in completely defined media using cells that had never been exposed to serum or other unidentified factors, and the rescue required neither insulin nor IGF1.

The effect of NDF differs from the survival effects of FGF2 described previously (Jessen et al., 1994; Gavrilovic et al., 1995) in at least two respects. First, NDF supports precursor survival for several days in defined medium without serum, whereas the effect of FGF2 turned out to be surprisingly transient under these conditions, lasting essentially for 1 day only. Evidently, longer-term survival in FGF2 depends on co-operativity between FGF2 and serum factors (Jessen et al., 1994). Second, NDF blocks death in medium containing a low concentration of insulin (10 nM) and no IGF1, i.e., in the apparent absence of type 1 IGF receptor stimulation (Sara and Hall, 1990). The action of FGF2, in contrast, crucially depends on a high concentration of IGF1.

When a half-maximal concentration of NDF was used, in combination with low doses of insulin for activation of the insulin receptor only (1 nM), the two factors synergized strongly in blocking cell death. However, even in the complete absence of both insulin and IGF1, higher concentrations of NDF supported survival similar to that seen in media containing low insulin or low insulin plus a high concentration of IGF1. Surprisingly, therefore, neither insulin receptor nor IGF receptor activation is necessary for NDF-mediated blockage of apoptosis in these cells.

Regulation of DNA Synthesis Differs between Schwann Cells and Precursors

In the present experiments, we have established an additional phenotype that distinguishes E14/15 precursors from E17 and older Schwann cells, namely the absence or presence of a mitogenic response to FGF2. We found previously that the $p75^{NGFR}$/L1-positive precursors in E14 nerves do not respond to FGF2 plus forskolin by stimulation of DNA synthesis (Gavrilovic et al., 1995). These cells, which are dividing at an appreciable rate in the nerve (Stewart et al., 1993), fall out of division during the first 20 hr in the culture dish, even in the presence of FGF2 and forskolin, although these factors effectively block apoptosis during the same period. We show here that the same applies to precursors from E15 nerves, while the L1-positive population from E17 nerves responds differently. Over 80% of these cells are Schwann cells on the basis of survival ability and S100 expression. They are dividing rapidly in vivo (Stewart et al., 1993), and this rate is maintained in the presence of FGF2 plus forskolin during the first 20 hr in vitro. Without FGF2 and forskolin, these cells survive but fall out of division during the same period. Cells removed from E18 and newborn nerves also respond to FGF2 plus forskolin by stimulation of DNA synthesis. The molecular basis for this difference in FGF responsiveness between E14/15 Schwann cell precursors on the one hand and E17 and older Schwann cells on the other now remains to be determined.

Schwann Cell Generation

S100 expression, survival requirements, and mitogenic response to FGF2 represent a diverse collection of apparently unrelated phenotypic features. Nevertheless, all three are subject to strong developmental regulation in the Schwann cell lineage. They change with a similar time course and during the same period of embryonic nerve development, i.e., between E14 and E18 with the major shift occurring between E15 and E17. This comprehensive phenotypic change can now be reproduced under defined

conditions in vitro: L1/NGFR-positive precursors removed from E14 nerves and exposed to NDFβ-2 for 1–4 days show a gradual alteration in all three parameters that follows a time course very similar to that seen in vivo. This suggests the existence of a program in E14 cells for coordinating the expression of a substantial number of functionally diverse genes. It is likely that this program is driven by NDF. Alternatively, it may unfold cell autonomously in cells in which NDF has blocked apoptosis. The resolution of these issues and the molecular identification of the underlying gene regulatory mechanisms represent important future lines of work.

Experimental Procedures

Antibodies

Rabbit antiserum to bovine S100 protein (Dakopatts A/C) was used at a dilution of 1:10,000. Monoclonal antibody (MAb) 192 IgG, which recognizes the low affinity $p75^{NGFR}$, was a gift from E. Johnson, Jr., and was used in the form of ascites fluid at a dilution of 1:100. MAb ASCS4, which in the rat recognizes the cell adhesion molecule L1 (Sweadner, 1983), was a gift from Dr. A. Furley. It was used in the form of cell culture supernatant. MAb against BrdU was a gift from M. Jones and Dr. D. Mason and was used at a dilution of 1:100. Rabbit antiserum to recombinant rat NDF (Wen et al., 1994) was used at a concentration of 1:50. Affinity-purified rabbit antiserum 1915 to recombinant human NDF was made and characterized by Dr. D. Wen, Amgen Center, and was used at a concentration of 5–10 μg/ml. Mouse MAb 5D6A against recombinant human NDF was made and characterized by Dr. D. Chang, Amgen Center, and was used at a dilution of 5–10 μg/ml. MAb K-15 to ErbB2 receptors and MAb C-18 to ErbB4 receptors were from Santa Cruz Biotechnology (used at 1 μg/ml). OX7 hybridoma cell line was from European Collection of Animal Cell Cultures. Rabbit anti-mouse immunoglobulin was from Dakopatts A/C. Goat anti-human antibody was from Sigma Chemical Company. Fluorescein conjugated to goat anti-rabbit immunoglobulin (Cappel Labs), absorbed with mouse immunoglobulin to remove cross-reacting antibodies, and tetramethyl rhodamine conjugated to goat anti-mouse immunoglobulin (Cappel Labs), absorbed with rabbit immunoglobulin to remove cross-reacting antibodies, were both used at a dilution of 1:100. Biotinylated sheep anti-rabbit and anti-mouse immunoglobulins and streptavidin–fluorescein were from Amersham International.

Other Materials

Transferrin, selenium, putrescine, triiodothyronine, thyroxine, dexamethasone, insulin, cytosine arabinoside, hyaluronidase, laminin, poly-L-lysine (molecular weight 300,000), and bovine serum albumin (BSA) were obtained from Sigma Chemical Company. Tissue culture petri dishes and 24-well plates were from Falcon. Protein A Sepharose column was from Pharmacia Biotech. Four-well plates were from Nunc Life Technologies. NGF was a gift from Dr. J. Winter; bFGF was from R&D Systems. Fetal calf serum was from Advanced Protein Products. Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), Ham's F12 medium, and trypsin were from GIBCO Laboratories. Collagenase was obtained from Worthington Biochemical Corporation. BrdU was from Boehringer Mannheim. Citifluor was obtained from City University (London).

Defined Medium

Defined medium used in this study was a modification of the medium of Bottenstein and Sato (1979). A 1:1 mixture of DMEM and Ham's F12 was supplemented with (final concentration in parentheses) transferrin (100 μg/ml), progesterone (60 ng/ml), putrescine (16 μg/ml), insulin (5.7 ng/ml, 10^{-9} M), IGF1 (100 ng/ml, 13 nM), thyroxine (0.4 μg/ml), selenium (160 ng/ml), triiodothyronine (10.1 ng/ml), dexamethasone (38 ng/ml), glucose (7.9 mg/ml), BSA (0.3 mg/ml), penicillin (100 IU/ml), streptomycin (100 IU/ml), and glutamine (2 mM). This medium is referred to as defined medium or defined medium without serum. In some experiments insulin and/or IGF1 were omitted from the medium; this is indicated in the text.

Cell Cultures

Cultures of Schwann cells and precursors were prepared essentially as described previously (Jessen et al., 1994; Gavrilovic et al., 1995). After dissociation and centrifugation, the cells were resuspended in defined medium, counted, and plated (at a density of 2000 cells per coverslip unless otherwise indicated) in a 10 or 20 μl drop on laminin-coated coverslips. After 3 hr, the cultures were topped up with 280 μl of defined medium. Coverslips were coated with poly-L-lysine and laminin as described previously (Jessen et al., 1994).

The survival assay has been described in detail previously (Gavrilovic et al., 1995; also see Jessen et al., 1994). In brief, 20 hr after plating the cells were fixed and immunolabeled with antibody 192 IgG against the $p75^{NGFR}$ or antibody ASCS4 against L1 (see below). The number of flattened immunopositive cells at this time point was counted in the fluorescence microscope and expressed as a percentage of the number of such cells on sister coverslips 3 hr after plating; this figure is referred to as percentage survival. The assay as described above is referred to as the 20 hr survival assay. In some experiments, survival was examined over longer periods; this is indicated in the text.

The BrdU assay of DNA synthesis was carried out by exposing cells to BrdU (20 μM) for periods of 1.5 hr followed by double immunolabeling using antibodies to L1 (see below) and BrdU. The percentage of L1-positive cells that also had BrdU-positive nuclei was taken to indicate the proportion of glial cells that synthesized DNA.

For identification of Schwann cell precursors, antibodies against L1 were used in most experiments, although the results were generally checked using antibodies against the $p75^{NGFR}$, which served as the antigenic marker for precursor identification in our earlier experiments (Jessen et al., 1994; Gavrilovic et al., 1995). Schwann cell precursors express cell surface L1 immunoreactivity (Jessen et al., 1994). In the present study, we carried out double-immunofluorescence experiments using ASCS4 antibody and 192 IgG antibody to detect L1 and NGFR, respectively, and class-specific fluorochrome-labeled anti-mouse second layer antibodies. All the L1-immunolabeled cells also expressed $p75^{NGFR}$. On the other hand, $p75^{NGFR}$ was also detectable on a few L1-negative cells. In cultures maintained in NDF (the conditions used in nearly all the experiments in the present paper), 3% of the $p75^{NGFR}$-positive cells were L1 negative after 20 hr in vitro, and the number of such cells after longer periods in culture was lower (data not shown). Some of the L1-negative/$p75^{NGFR}$-positive cells showed neither the flattened morphology nor the group-forming tendency characteristic of the very great majority of cells in the precursor cultures, but tended instead to occur singly on the coverslip; furthermore, preliminary tests indicated that these cells did not acquire significant S100 expression with time in culture. It is possible that this small subpopulation of $p75^{NGFR}$-positive cells does not belong to the glial lineage. However that may be, we have preferred, in the present work, not to make our observations on the total $p75^{NGFR}$-positive population but rather to confine them, wherever practicable, to the slightly smaller and more homogenous population of cells that express both L1 and $p75^{NGFR}$ and take part in forming flattened cellular groups. For practical reasons, $p75^{NGFR}$ was nevertheless used to identify precursors in some double-labeling experiments; this is indicated in the text.

For culturing neurons, a pellet of DRG cells was prepared as described elsewhere (Jessen et al., 1994). The cells were then resuspended in 6 ml of DMEM plus 10% fetal calf serum or defined medium, and the suspension was transferred to a 90 mm plastic dish coated with antibodies against Thy 1.1 in DRG cell suspension. (Thy 1.1 is present on both neurons and fibroblasts, but not on glial cells.) After 2–3 min at 37°C, the dish was washed 4–5 times very quickly with DMEM plus 10% fetal calf serum or defined medium. The neurons were then streamed off with defined medium containing 50 ng/ml NGF and plated in 4-well poly-L-lysine-coated plastic plates at a density of 100,000–150,000 neurons/well. The volume of culture medium per well was 300 μl, and 95% of the cells in the cultures at 20 hr were neurons. The medium was changed at 20 hr, and conditioned medium was collected at 48 and 72 hr after plating only.

To coat with anti-Thy 1.1, the dish was first incubated with 6 ml of Tris buffer solution (50 mM; pH 9.5) containing rabbit anti-mouse immunoglobulin (38 μg/ml final concentration) for 18 hr at 4°C. It was then washed 3 times with phosphate-buffered saline (PBS) and incubated with a mixture of OX7 antibodies (Mason and Williams, 1980)

in the form of culture supernatant (4 ml), MEM–HEPES (2 ml), and BSA (400 μl at 35 mg/ml) for 1–4 hr at room temperature. The dish was washed 3 times with PBS and used immediately.

In replating assays for monitoring precursor maturation, cells from nerves at various ages (see Results) were plated at a density of 12,000 cells per well in 50 μl of defined medium on laminin-coated, poly-L-lysine-coated wells. After 3 hr, the medium was withdrawn and replaced with 300 μl of defined medium containing the appropriate concentration of NDFβ-2. After 1–4 days in vitro, the cells were rinsed once with versene and incubated for 5 min with 300 μl of versene plus 100 μl of enzyme cocktail at 37°C. The cells were triturated off the wells and removed to a centrifuge tube containing defined medium, spun at 1000 rpm for 10 min, and resuspended in defined medium. The cells were replated at a density of 2,000 cells in 20 μl on laminin-coated coverslips. After 3 hr, the cultures were topped with defined medium for the survival assay, with defined medium containing 3 ng/ml bFGF and 5 μM forskolin for the cell division assay, or fixed for examination of S100 expression.

Construction and Expression of a Secreted Soluble ErbB4 Receptor

To construct a soluble ErbB4 receptor–immunoglobulin chimeric DNA, the expression vector CDM7 (Invitrogen) bearing the extracellular portion of ErbB4 fused in frame to an Fc portion (hinge, CH2, and CH3) of a human immunoglobulin γ-1 was used. Essentially, the parental CDM7 plasmid was digested with BamHI and EcoRI to allow fusion of the Fc portion with the extracellular domain of ErbB4 (denoted IgB4). The extracellular domain of ErbB4 was amplified by using the polymerase chain reaction (30 cycles of 1.5 min at 96°C, 2 min at 52°C, and 3 min at 72°C). The amplifed extracellular domain was purified and digested with BamHI and EcoRI and inserted into the appropriate sites in the expression vector. The upstream and downstream oligonucleotide primers of ErbB4 had the following sequences: 5′-CGCCGGGAATTCCAAAAAATGAAGCCGGCGAC-3′ and 5′-CCCGGGGATCCCTAGCATGTTGTGGTAAAGTGG-3′, respectively. The built-in cloning sites are underlined. Nucleotide sequencing confirmed the integrity of the open reading frames of the chimeric cDNA and also partially verified correct sequences. For electroporation of HEK 293 cells, 10 μg of the resulting IgB4 plasmid together with 0.5 μg of pSV2/neo was mixed with 2×10^6 cells in 0.8 ml of DMEM. The electroporation was carried out with a Bio-Rad gene pulser using voltage and capacitance settings of 270 V and 960 μF, respectively. Individual clones were selected with G418 (800 μg/ml) and maintained in DMEM with 10% fetal calf serum. The conditioned media were assayed with a goat anti-human antibody to select positive clones that secreted the fusion proteins. Media conditioned by a selected cell clone were then purified using protein A Sepharose column. Protein concentration was estimated by gel staining and comparison with myosin.

Immunohistochemistry

Immunolabeling of S100, L1, NGFR, and BrdU is described elsewhere (Jessen et al., 1994). Fixation for ErbB2 and ErbB4 was 4% paraformaldehyde (20 min) followed by PBS wash and then 0.1% Triton X-100 (20 min). ErbB2 and ErbB4 immunoreactivity was visualized using the biotin–streptavidin method. Fixation for NDF was 2% paraformaldehyde (7 min). NDF immunoreactivity was visualized using the biotin–streptavidin method. All cells fixed by 2% paraformaldehyde prior to immunostaining were fixed with 4% paraformaldehyde at the end of the immunolabeling.

Controls for Immunohistochemistry

In every case, omission of the first antibody layer abolished any immunolabeling. Absorption of the anti-ErbB2 antibody with ErbB2 control peptide, but not with ErbB4 peptide, abolished the immunolabeling. Similarly, absorption of the anti-ErbB4 antibody with ErbB4 control peptide, but not with ErbB2 peptide, abolished labeling. Absorption of antiserum to NDF with NDFβ-2, but not with FGF2, abolished immunolabeling of DRG neurons.

Quantification

All quantitative results are based on a minimum of three separate experiments (unless stated otherwise). Each determination within an experiment is based on counts from three coverslips in the great majority of cases, but occasionally on counts from two or four coverslips. Error bars on graphs indicate SEM. In all survival assays, survival is expressed as "percentage survival"; this term is defined above.

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References

Ben-Baruch, N., and Yarden, Y. (1994). Neu differentiation factors: a family of alternatively spliced neuronal and mesenchymal factors. Proc. Soc. Exp. Biol. Med. *206*, 221–227.

Bottenstein, J. E., and Sato, G. H. (1979). Growth of rat neuroblastoma cell line in serum-free supplemented medium. Proc. Natl. Acad. Sci. USA *76*, 514–517.

Bray, G. M., Rasminsky, M., and Aguayo, A. J. (1981). Interactions between axons and the sheath cells. Annu. Rev. Neurosci. *4*, 127–162.

Brockes, J. P., Fields, K. L., and Raff, M. C. (1979). Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. Brain Res. *165*, 105–118.

Bunge, M. B., Clark, M. B., Dean, A. C., Eldridge, C. F., and Bunge, R. P. (1990). Schwann cell function depends upon axonal signals and basal lamina components. Ann. NY Acad. Sci. *580*, 281–287.

Cohen, J. A., Yachnis, A. T., Arai, M., Davis, J. G., and Scherer, S. S. (1992). Expression of the *neu* proto-oncogene by Schwann cells during peripheral nerve development and Wallerian degeneration. J. Neurosci. Res. *31*, 622–634.

Culouscou, J.-M., Plowman, G. D., Carlton, G. W., Green, J. M., and Shoyab, M. (1993). Characterization of a breast cancer cell differentiation factor that specifically activates the HER4/p180^{erbB4} receptor. J. Biol. Chem. *268*, 18407–18410.

Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993). ARIA, a protein that stimulates acetylcholine receptor synthesis, is a member of the Neu ligand family. Cell *72*, 801–815.

Gavrilovic, J., Brennan, A., Mirsky, R., and Jessen, K. R. (1995). Fibroblast growth factors and insulin growth factors combine to promote survival of rat Schwann cell precursors without induction of DNA synthesis. Eur. J. Neurosci. *7*, 77–85.

Goodearl, A. D. J., Davis, J. B., Mistry, K., Minghetti, L., Otsu, M., Waterfield, M. D., and Stroobant, P. (1993). Purification of multiple forms of glial growth factor. J. Biol. Chem. *268*, 18095–18102.

Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., et al. (1992). Identification of heregulin, a specific activator of p185*erb*B2. Science *256*, 1205–1210.

Jessen, K. R., and Mirsky, R. (1992). Schwann cells: early lineage, regulation of proliferation and control of myelin formation. Curr. Opin. Neurobiol. *2*, 575–581.

Jessen, K. R., Mirsky, R., and Morgan, L. (1987). Axonal signals regulate the differentiation of non-myelin-forming Schwann cells: an immunohistochemical study of galactocerebroside in transected and regenerating nerves. J. Neurosci. *7*, 3362–3369.

Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y., and Gavrilovic, J. (1994). The Schwann cell precursor and its fate: a study of cell death and differentiation during gliogenesis in rat embryonic nerves. Neuron *12*, 509–527.

Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., et al. (1993). Glial growth factors are alternatively spliced erbB2 ligands expressed in the nervous system. Nature *362*, 312–318.

Mason, D. W., and Williams, A. F. (1980). The kinetics of antibody binding to membrane antigens in solution and at the cell surface. Biochem. J. *187*, 1–20.

Meyer, D., and Birchmeier, C. (1994). Distinct isoforms of neuregulin are expressed in mesenchymal and neuronal cells during mouse development. Proc. Natl. Acad. Sci. USA *91*, 1064–1068.

Orr-Urtreger, A., Trakhtenbrot, L., Ben-Levy, R., Wen, D., Rechavi, G., Lonai, P., and Yarden, Y. (1993). Neural expression and chromosomal mapping of Neu differentiation factor to 8p12-p21. Proc. Natl. Acad. Sci. USA *90*, 1867–1871.

Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben-Levy, R., and Yarden, Y. (1992). Isolation of the *neu*/HER-2 stimulatory ligand: a 44 kd glycoprotein that induces differentiation of mammary tumor cells. Cell *69*, 205–216.

Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. (1993). Cell-type specific interaction of Neu differentiation factor (NDF/heregulin) with Neu/HER-2 suggests complex ligand-receptor relationships. EMBO J. *12*, 961–971.

Pinkas-Kramarski, R., Eilam, R., Spiegler, O., Lavi, S., Liu, N., Chang, D., Wen, D., Schwartz, M., and Yarden, Y. (1994). Brain neurons and glial cells express Neu differentiation factor/heregulin: a survival factor for astrocytes. Proc. Natl. Acad. Sci. USA *91*, 9387–9391.

Plowman, G. D., Culouscou, J.-M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. (1993a). Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA *90*, 1746–1750.

Plowman, G. D., Green, J. M., Culouscou, J.-M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993b). Heregulin induces tyrosine phosphorylation of HER4/p180^{erbB4}. Nature *366*, 473–475.

Raff, M. C., Abney, E., Brockes, J. P., and Hornby-Smith, A. (1978). Schwann cell growth factors. Cell *15*, 813–822.

Ratner, N., Bunge, R. P., and Glaser, L. (1985). A neuronal heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. J. Cell Biol. *101*, 744–754.

Sara, V. R., and Hall, K. (1990). Insulin-like growth factors and their binding proteins. J. Am. Physiol. Soc. *70*, 591–614.

Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P., and Anderson, D. J. (1994). Glial growth factor restricts mammalian neural crest stem cells to a glial fate. Cell *77*, 349–360.

Sliwkowski, M. X., Schaefer, G., Aktia, R. W., Lofgren, J. A., Fitzpatrick, U. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L. (1994). Coexpression of ErbB-2 and ErbB-3 protein reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. *269*, 14661–14665.

Stewart, H. J. S., Morgan, L., Jessen, K. R., and Mirsky, R. (1993). Changes in DNA synthesis rate in the Schwann cell lineage *in vivo* are correlated with the precursor–Schwann cell transition and myelination. Eur. J. Neurosci. *5*, 1136–1144.

Sweadner, K. J. (1983). Post-translational modification and evoked release of two large surface proteins of sympathetic neurons. J. Neurosci. *3*, 2504–2517.

Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994). ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms. J. Biol. Chem. *269*, 25226–25233.

Vartanian, T., Corfas, G., Li, Y., Fischbach, G. D., and Stefansson, K. (1994). A role for the acetylcholine receptor-inducing protein ARIA in oligodendrocyte development. Proc. Natl. Acad. Sci. USA *91*, 11626–11630.

Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Ben Levy, R., et al. (1992). Neu differentiation factor: a transmembrane glycoprotein containing an EGF domain and an immunoglobulin homology unit. Cell *69*, 559–572.

Wen, D., Suggs, S. V., Karunagaran, D., Liu, N., Cupples, R. L., Luo, Y., Janssen, A. M., Ben-Baruch, N., Trollinger, D. B., Jacobsen, V. L., et al. (1994). Structural and functional aspects of the multiplicity of Neu differentiation factors. Mol. Cell. Biol. *14*, 1909–1919.