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Basic Fibroblast Growth Factor (bFGF) acts intracellularly to cause the transdifferentiation of avian neural crest-derived Schwann cell precursors into melanocytes

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SUMMARY

We previously found that cultured neural crest-derived cells from embryonic quail peripheral nerves, which consist mostly of Schwann cell precursors, gave rise to melanocytes following treatment with basic fibroblast growth factor (bFGF) or 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Here, we show that antisense deoxyoligonucleotides targeted against two regions of the bFGF mRNA transcript blocked this TPA-induced transdifferentiation of Schwann cell precursors. Neither sense nor scrambled antisense control oligonucleotides had any effect in this regard. TPA increased bFGF protein expression in cell lysates but not in conditioned media from these cultures, and this expression was localized to the nucleus and cytoplasm. Furthermore, bFGF-neutralizing antibodies and inositolhexakisphosphate ($InsP_6$) both inhibited pigmentation caused by exogenous bFGF, but had no affect on TPA-induced melanogenesis, suggesting that bFGF is not released by these cells. These data indicate that bFGF is necessary for the TPA-induced transdifferentiation of Schwann cell precursors into melanocytes and that bFGF acts via an intracrine mechanism.

Key words: neural crest, fibroblast growth factor, melanocyte, Schwann cell, intracrine, phorbol ester

INTRODUCTION

The neural crest (NC) is a transitory structure in vertebrate embryos arising from cells of the neuroepithelium as it folds to form the neural tube during neurulation. NC cells migrate from the neural tube and localize at characteristic sites in the periphery, giving rise to diverse cellular phenotypes. These include melanocytes of the integument and iris, Schwann cells, sensory and autonomic neurons, neurosecretory cells of the adrenal medulla, and connective tissue of the head and face (see Le Douarin, 1982). It has been suggested that at least some NC cells become committed to these phenotypes via a series of developmental restrictions, which progressively limit their potential fates (Anderson, 1989; Weston, 1991). In support of this notion, various studies have implicated the transient existence of partially restricted subpopulations of NC-derived cells, which undergo subsequent commitment in response to environmental cues. The NC-derived sympathoadrenal progenitor, for example, is believed to arise during late embryogenesis and has the capability of giving rise to sympathetic neurons or to pheochromocytes of the adrenal medulla, but not other NC derivatives (Anderson and Axel, 1986; Landis and Patterson, 1981; Carnahan and Patterson, 1991). This hypothesis is based on observations that cells isolated from neonatal rat sympathetic ganglia or embryonic rat adrenal medullae can give rise to neurons in the presence of nerve growth factor (NGF) or to chromaffin cells in the presence of glucocorticoids (Aloe and Levi-Montalcini, 1979; Doupe et al., 1985; Anderson and Axel, 1986; Seidl and Unsicker, 1989).

An analogous bipotent intermediate in the NC cell lineage, which can give rise to either Schwann cells or melanocytes, has been shown to arise in clones of avian NC cells in culture and in vivo (Dupin et al., 1990; Bronner-Fraser and Fraser, 1989). This 'melanocyte/Schwann cell progenitor' was first proposed by Nichols and Weston (1977), who demonstrated that explants of various NC derivatives from 'early' (E4-E6) avian embryos, including peripheral nerves, gave rise to melanocytes under permissive conditions, whereas explants from older embryos did not. These peripheral nerve explants are believed to consist largely of Schwann cell precursors (i.e., NC-derived cells, which would normally give rise to Schwann cells, but which are not yet fully committed to this fate). We later found that cells with latent melanogenic potential were present in embryonic quail peripheral nerves and other NC derivatives in these older embryos. These cells underwent a transdifferentiation into pigment cells, but not other NC derivatives,

following treatment with the tumor-promoting phorbol ester drug 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (Ciment et al., 1986; see also Kanno et al., 1987). These data suggest that TPA can reverse the developmental restriction of melanogenesis that normally occurs during early NC development, causing the transdifferentiation of cells, which would normally have given rise to Schwann cells, into melanocytes (Ciment, 1990).

More recently, we found that basic fibroblast growth factor (bFGF), but not various other growth factors, could also reverse the developmental restriction of melanogenesis in early avian Schwann cell precursors (Stocker et al., 1991). When added to cultures of embryonic day 7 (E7) quail peripheral nerves or dorsal root ganglia (DRG), optimal concentrations of bFGF induced melanogenesis in 20-60% of the cultures, as opposed to 90-100% of the cultures treated with TPA. Basic FGF is a polypeptide growth factor expressed in numerous tissues, which acts as a mitogen and differentiation factor for various mesoderm- and neurectoderm-derived cells (for reviews, see Haynes, 1988; Rifkin and Moscatelli, 1989; Gospodarowicz, 1990). This growth factor is known to be present in avian embryos, moreover, at the relevant stages and locations to influence the fate of migrating NC cells (Kalcheim and Neufeld, 1990), including the melanocyte/Schwann cell progenitors, implicating it as a potential extracellular signaling molecule in NC development. This notion is consistent with observations that bFGF can act as a survival factor for a subpopulation of non-neuronal NC cells (Kalcheim, 1989) and can promote neurogenesis in avian NC progenitors (Brill et al., 1992) and immortalized rat sympathoadrenal precursors (Birren and Anderson, 1990). Some migrating NC-derived cells, moreover, transiently express FGF receptor mRNA in vivo (Heuer et al., 1990).

It is unclear, however, whether all of the reported effects of bFGF can be explained via its release into the extracellular space. The bFGF protein lacks, for example, a hydrophobic secretory signal sequence (Abraham et al., 1986) and only a few cell types have been shown to secrete it, presumably via a pathway independent of the endoplasmic reticulum-Golgi complex (Vlodavsky et al., 1987; Kandel et al., 1991; Mignatti and Rifkin, 1991; Mignatti et al., 1992; Shain et al., 1992). Several groups, however, have reported that bFGF is concentrated intracellularly, especially within the nucleus of various cell types (Florkiewicz et al., 1991; Tesler and Neufeld, 1990; Bugler et al., 1991; Woodward et al., 1992) and that its secretion may not be necessary to elicit its biological activity (Neufeld et al., 1988). Although the function of such nuclear bFGF is unclear, there is reason to believe that it may play a direct role in transcriptional control (Bouche et al., 1989; Baldin et al., 1990; Quarto et al., 1991a; Nakanishi et al., 1992), leading to the suggestion that bFGF can act, in at least some cells, via an 'intracrine' pathway involving intracellular signaling without release [for review, see Logan (1990)].

The mechanisms governing bFGF expression are also poorly understood (see Gospodarowicz, 1990). The putative bFGF promoter, however, is known to contain a potential TPA-responsive element (Shibata et al., 1991) and TPA has been shown to induce bFGF mRNA expression in a variety of cell types (Murphy et al., 1988a; Bikfalvi et al., 1990; Weich et al., 1991; Lowe et al., 1992). It is possible, therefore, that bFGF may mediate the effects of TPA in our experimental system. In this paper, we investigate the role of bFGF in the TPA-induced transdifferentiation of Schwann cell precursors in peripheral nerve explant cultures into melanocytes. We find that TPA augments the expression of bFGF protein in these peripheral nerve cultures, and that bFGF expression is necessary for this transdifferentiation to occur. We also report that bFGF is not released by these cells, indicating that bFGF is probably acting via an intracrine mechanism. These results support the notion that bFGF may act as an intracellular signaling molecule and may provide insights into the intracellular mechanisms regulating the commitment of NC-derived melanocyte/ Schwann cell progenitors.

MATERIALS AND METHODS

Embryos

Fertilized quail eggs (*Coturnix coturnix japonica*) were obtained from the Poultry Science Department at Oregon State University (Corvallis, OR). Eggs were incubated in a humidified incubator at 38°C and the embryos were staged using the criteria of Hamburger and Hamilton (1951).

Reagents

Recombinant human bFGF (rh-bFGF) was obtained from Collaborative Research (Lexington, MA). Insulin, transferrin, selenium, α-MSH, L-glutamine, penicillin, streptomycin, CHAPS, inositol-hexakisphosphate ($InsP_6$) and TPA were all purchased from Sigma Chemical Co. (St Louis, MO). Anti-human bFGF monoclonal antibodies DE6 (Reilly et al., 1989), anti-human bFGF monoclonal antibody 148.6.1.1 and anti-P_0 monoclonal antibody 1E8 (Bhattacharyya et al., 1991) were the generous gifts of Drs Janet Gross (E. I. Dupont), Charles Hart (Zymogenetics) and Eric Frank (Univ. Pittsburgh), respectively. Conditioned medium containing the NC-specific monoclonal antibody, HNK-1 (Bronner-Fraser, 1986), was harvested from HNK-1 hybridoma cells which were obtained from the American Type Culture Collection.

Oligonucleotides corresponding to codons spanning the human bFGF translation initiation site (ATG) and the first splice-donor acceptor site (codon 58) were synthesized by Research Genetics (Huntsville, AL). Oligonucleotides (Table 1) were solubilized in sterile isotonic phosphate-buffered saline (PBS) and added directly to peripheral nerve explants soon after dissection. Phosphorothioate-modified oligonucleotides were used at a final concentration of 10 μM, while unmodified oligonucleotides were used at 50 μM, as described previously (Morrison, 1991).

Dissection and tissue culture

Peripheral nerves were dissected from the brachial, thoracic and lumbosacral levels of stage 32-33 quail embryos and cultured as explants in either 96-well or 60 mm Primaria plates (Falcon Plastics, Ventura, CA) in a humidified 5% CO_2-95% air atmosphere at 37°C. Peripheral nerve explants were used because previous studies had shown that explant cultures produced less variable pigmentation than dissociated cell cultures (unpublished observations). Complete medium ('CM') consisted of Hepes-buffered Ham's F-12 medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 0.03% L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 20% heat-inactivated fetal bovine serum (Hyclone, Logan, UT). For the defined medium ('DM'), serum was replaced with 1 mg/ml bovine serum albumin, 5 ng/ml selenium, 1 μg/ml transferrin, 1 μg/ml insulin and 100 ng/ml α-MSH. Culture

media were further supplemented with various combinations of rh-bFGF, oligonucleotides and TPA (made from a 1000× stock solution in ethanol). Culture medium without TPA contained 0.1% ethanol as a vehicle control. Half of the medium in each culture was replaced with fresh medium and supplements (except oligonucleotides) after 5 days, unless otherwise indicated.

Melanogenesis assay

Live cultures were examined at various times for the presence of pigmented cells, as described previously (Stocker et al., 1991) using a Nikon TMS inverted microscope with bright-field illumination. Wells were scored as positive when they contained one or more pigmented cells. Care was taken to distinguish melanocytes (i.e., cells with a dendritic morphology containing dark, ovoid melanosomes) from brownish-yellow degenerating cells containing irregularly shaped granulations. 'Percent pigmentation' was calculated by dividing the number of positive wells by the total number of wells. Each data point represents observations from 48 peripheral nerve cultures, unless otherwise noted. Statistical analysis involved the Newman Keuls multi-comparison test, with $P<0.05$ defined as statistically significant.

Preparation of cell lysates and conditioned media

Cultures of 80-100 peripheral nerve explants were washed three times in ice-cold PBS, then scraped from dishes in 50 μl of 20 mM Tris-HCl pH 7.0 (4°C) containing 2.0 M NaCl, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 0.2 mM phenylmethylsulfonyl fluoride and 100 μg/ml of leupeptin, pepstatin and aprotonin (Boehringer-Mannheim, Indianapolis, IN). The cell suspension was then sonicated for 1 minute, clarified by centrifugation at 14,000 ***g*** for 2 hours at 4°C, and stored at −80°C. Conditioned media from these cultures were applied to heparin-Affigel columns (Biorad, Richmond, CA). The columns were washed with 0.6 M NaCl and heparin-bound proteins were eluted with 2.0 M NaCl. Samples were then concentrated approximately 100-fold in a speed vacuum apparatus and dialyzed against 20 mM Tris-HCl (pH 7.0) for 16 hours. Protein concentrations in cell lysates and column eluates were determined using the method of Bradford (1976).

Gel electrophoresis and bFGF protein immunoblots

Protein bands were resolved by SDS-polyacrylamide (15%) gel electrophoresis using standard methods (Laemmli, 1970). Proteins were then transferred to nitrocellulose by electroblotting and non-specific binding sites were blocked by treatment in 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20, 1% bovine serum albumin and 10% normal horse serum (TBST) for 30 minutes at room temperature. Blots were then incubated for 1 hour at room temperature in TBST with either a 1:5000 dilution of the anti-bFGF monoclonal antibody DE6 or a 1:2500 dilution of the anti-bFGF monoclonal antibody 148.6.1.1. Blots were then washed three times for 5 minutes each with TBST, incubated for 30 minutes with a 1:2500 dilution of a biotinylated horse anti-mouse immunoglobulin antibody (Vector Laboratories, Burlingame, CA) in TBST, washed again, and then incubated for 30 minutes in strepavidin-HRP. Immunoreactive bands were visualized using an ECL enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). Negative controls involved the exclusion of primary antibodies or preabsorbing anti-bFGF antibodies with excess rh-bFGF prior to the incubation steps. The molecular weights of immunoreactive bands were determined by comparison with pre-stained 'low molecular weight' standards (Biorad).

Immunostaining and photomicroscopy

Peripheral nerve explant cultures were first washed with PBS, fixed in 90% ethanol (0°C):5% glacial acetic acid for 30 minutes and then washed again with PBS. To block non-specific immunostaining, cultures were incubated at room temperature for 1 hour in 10% serum in 'Buffer A,' consisting of 100 mM Tris buffer (pH 7.8), containing 150 mM NaCl, 0.5% Triton X-100, 0.05% sodium azide. HNK-1 immunostaining was performed by incubating cultures overnight at 4°C with HNK-1 conditioned medium diluted 1:20 in Buffer A, followed by secondary incubation with fluorescein isothiocyanate-labeled rabbit anti-mouse IgM antisera (1:200 dilution; Cappel, Durham, NC). Fluorescently labeled cultures were then mounted in a 1:1 solution of PBS and glycerol containing 1 μg/ml *p*-phenylenediamine. Basic FGF and P_0 immunostaining was performed by incubating cultures overnight at 4°C with either a 1:150 dilution of anti-bFGF antibody DE6 or a 1:250 dilution of anti-P_0 antibody 1E8 in Buffer A, and then processing these cultures using a Vectastain kit (Vector Laboratories), following the manufacturer's instructions. Negative control experiments involved either leaving out primary antibody or, in the case of bFGF immunostaining, DE6 was pre-adsorbed with 1 μg/ml of rh-bFGF. Photomicrographs were taken with a Zeiss axioplan microscope equipped with an Olympus photomicroscopy system.

Bromodeoxyuridine assay

The effects of TPA and bFGF on peripheral nerve cell proliferation were determined using a bromodeoxyuridine (BrdU) cell proliferation kit from Amersham. Briefly, individual peripheral nerve explants were cultured for 47 hours in the presence or absence of either TPA or bFGF. These cultures were then incubated at 37°C for 1 hour with a 10:1 mixture of 5-bromo-2′-deoxyuridine and 5-fluoro-2′-deoxyuridine (a thymidilate synthetase inhibitor) prior to fixation in 90% ethanol (0°C):5% glacial acetic acid for 30 minutes. Immunostaining for BrdU incorporated into nuclei was performed according to the manufacturer's instructions using cobalt and nickel to intensify the diaminobenzidine reaction product. Cells were then lightly counterstained with 0.1% light-green yellowish for 5 minutes and examined under a Nikon TMS inverted microscope with bright-field illumination. The percentage of $BrdU^+$ peripheral nerve cells was determined by dividing the number of darkly stained nuclei by the total number of cells in each culture.

RESULTS

Characterization of embryonic peripheral nerve explant cultures

Although adult peripheral nerves are known to contain primarily NC-derived Schwann cells and some mesoderm-derived perineurial fibroblasts (Bunge et al., 1989), the precise cellular composition of peripheral nerve explant cultures from stage 32-33 (E6.5-E7) quail embryos is unclear. To characterize the cellular composition of this tissue in culture, individual peripheral nerve explants were grown for 4 days, and then fixed and immunostained using either the NC-specific antibody HNK-1 (Bronner-Fraser, 1986) or the Schwann cell-specific monoclonal antibody 1E8 (Bhattacharyya et al., 1991). Fig. 1A is a phase-contrast photomicrograph from one such culture and shows that the majority of the cells from these explants have a flattened morphology with the relatively small nucleus characteristic of early Schwann cells (e.g., Jessen and Mirsky, 1992). Fig. 1B shows HNK-1 immunostaining in this same microscopic field, indicating that the vast majority of cells in E7 quail peripheral nerve cultures express this NC cell trait. The small numbers of $HNK\text{-}1^-$ cells in these cultures have relatively larger nuclei than the $HNK\text{-}1^+$ cells and are presumably perineurial fibroblasts. Fig. 1C represents a similar

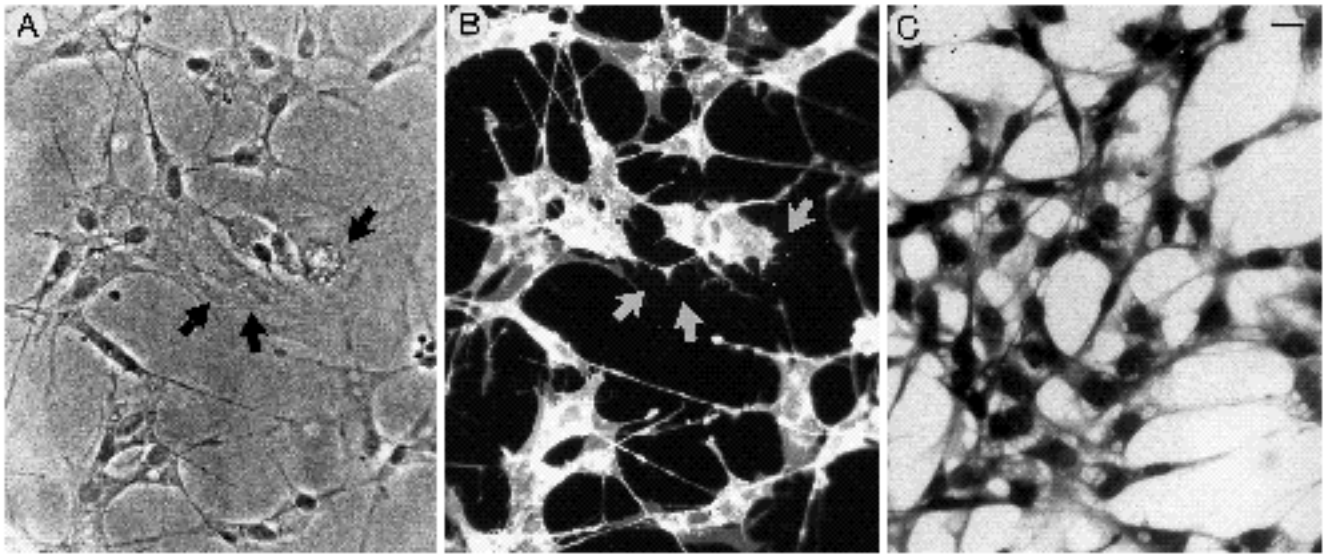

Fig. 1. Immunostaining studies of the NC marker, HNK-1, and the Schwann cell marker, P_0, in explant cultures of E7 quail (stage 32-33) peripheral nerves maintained for 4 days in CM. (A) Phase-contrast photomicrograph of a typical peripheral nerve explant culture. (B) Fluorescence photomicrograph of HNK-1 immunostaining in this same culture. Note that the majority of the cells in this culture are HNK-1^+, and that the few HNK-1^- cells (at the arrows) possess large nuclei and a fibroblast-like morphology. (C) Bright-field photomicrograph of P_0 immunoperoxidase staining in a similar culture to that seen in A and B. Note that all of the cells in this culture are P_0^+, suggesting that the vast majority of the cells in these peripheral nerve cultures are part of the Schwann cell lineage. The bar in the upper right corner represents 20 μm.

culture immunostained for the Schwann cell marker P_0 using peroxidase-labeled secondary antibodies instead of fluorescence antibodies to increase sensitivity. Note that in this particular culture all of the cells are P_0^+. Quantitative analyses of 12 such cultures shows that nearly all of the cells (>95%) typically stained positive for HNK-1 and that, in 83% (10/12) of these cultures, all of the cells were P_0^+. These data indicate that the vast majority of the cells in these peripheral nerve cultures are NC-derived cells of the Schwann cell lineage.

Effects of TPA on bFGF expression

TPA has previously been shown to increase bFGF mRNA levels in various cell types. To determine whether TPA influenced bFGF expression in peripheral nerve explants, cultures were grown for various periods of time in the presence or absence of 1.0 μM TPA, and then cell lysates and conditioned medium were assayed for bFGF, using protein immunoblot analysis. Fig. 2A shows that lysates of peripheral nerve cells cultured in CM alone contained low levels of three different relative molecular mass species of bFGF [approximately 17, 20, and 23 $\times 10^3$ M_r] at 24 hours and slightly higher levels at 48 hours (lanes 4 and 6, respectively). TPA had no significant effects on bFGF expression after 24 hours in culture (compare lanes 4 and 5), but caused an approximately 4-fold increase in levels of the various bFGF isoforms after 48 hours (compare lanes 6 and 7), as determined by densitometry. In a separate experiment, bFGF protein levels were found to return to basal levels by about 72 hours (not shown). In contrast to these cell lysates, bFGF was never detected in the 100-fold concentrated conditioned medium from these same cultures (Fig. 2B; lanes 4-7). These experiments were performed a total of five times using either DE6 or 148.6.1.1 antibodies with qualitatively similar results. Control experiments using DE6 antibodies preabsorbed with an excess of rh-bFGF showed no detectable bands in similar protein immunoblots (not shown).

Serum has also been reported to induce bFGF mRNA expression and may be necessary for bFGF protein expression in various cell types in culture (Murphy et al., 1988b). To determine whether the basal levels of bFGF seen in lanes 4 and 6 of Fig. 2A may have been induced by the serum present in CM, peripheral nerve explants were grown in a defined ('DM') serum-free culture medium (Stocker et al., 1991) in the presence and absence of TPA, and then assayed for bFGF immunoreactivity. As shown in lane 8 of Fig. 2A, no bFGF could be detected in cell lysates prepared from these cultures, even in the presence of TPA (lane 9), suggesting that some component(s) of fetal bovine serum can contribute to, and is necessary for, the induction of bFGF expression in these cultured peripheral nerve cells.

Effects of bFGF-antisense oligonucleotides on TPA-induced transdifferentiation

To determine if bFGF protein expression was necessary for TPA-induced melanogenesis in peripheral nerve cells, cultures were treated with TPA in the presence and absence of antisense oligonucleotides corresponding to two distinct regions of the bFGF mRNA transcript (Table 1), and then examined at various times for the presence of melanocytes and for bFGF expression. Fig. 3A represents the time course of pigmentation under these various culture conditions from a representative experiment; Fig. 3B presents the statistical analysis at the end of 10 days from four such experiments. As shown in these panels, bFGF antisense oligonucleotides

'AS1' and 'AS2' delayed the initial appearance of pigment cells by 24-48 hours and caused a statistically significant 40% reduction of TPA-induced pigmentation after 10 days. In contrast, TPA-induced pigmentation was not significantly influenced in cultures incubated with sense ('S2') and scrambled antisense oligonucleotides ('SAS2'). Cell viability did not appear to be affected in cultures containing oligonucleotides (data not shown). Furthermore, the numbers of melanocytes in wells containing pigmented cells (typically 5-25% of the cells) did not vary with the different treatment conditions. Interestingly, addition of phosphorothioate-modified antisense oligonucleotides ('PAS1'), which are presumably less vulnerable to nuclease degradation (Marcus-Sekura et al., 1987), did not change the levels of pigmentation as compared to cultures treated with unmodified oligonucleotides ('AS1').

Table 1. Oligonucleotides and their corresponding location within the bFGF mRNA transcript

Sequence name	Oligonucleotide	Location	Sequence
AS1	Antisense	Start site	5′-GGC-TGC-CAT-GGT-CCC-3′
AS2	Antisense	Codon 58	5′-TAG-CTT-GAT-GTG-AGG-3′
S2	Sense	Codon 58	5′-CCT-CAC-ATC-AAG-CTA-3′
SAS2	Scrambled antisense	Codon 58	5′-ATC-GGG-TTG-TGG-TAA-3′

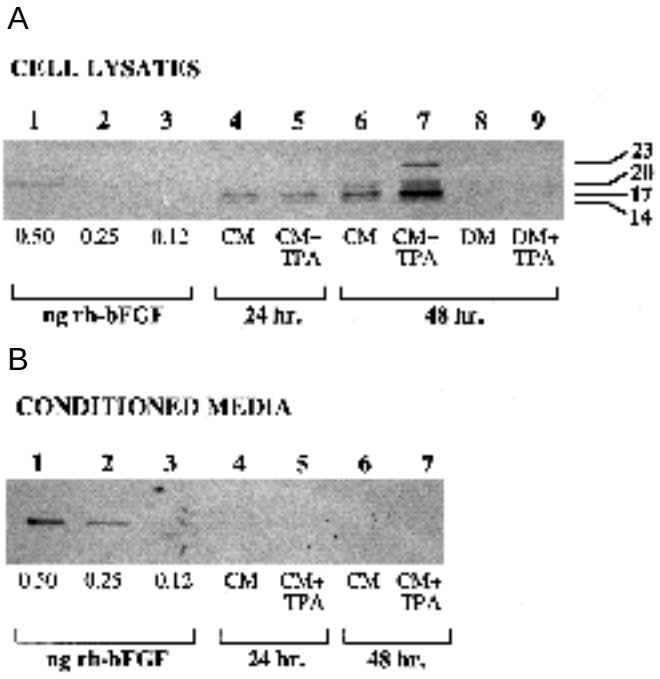

Fig. 2. Effects of TPA and serum on the levels of the various bFGF isoforms in peripheral nerve explant cultures. Explants of stage 32-33 quail embryo peripheral nerves were cultured in serum-containing ('CM') or serum-free ('DM') medium for either 24 or 48 hours in the presence or absence of TPA (1.0 μM TPA). At these times, the cells and conditioned media were harvested and analyzed for the presence of bFGF protein using immunoblot analysis. (A) Basic FGF expression in cell lysates. Note that explants cultured in CM expressed three molecular weight isoforms of bFGF (approximately 17, 20, and 23×10^3 M_r), and that the levels of these isoforms, and in particular the 23×10^3 M_r form, increased by 48 hours (compare lanes 4 and 6). A smaller molecular weight band at approximately 14×10^3 M_r was also present, and may represent degraded bFGF. Also note that TPA augmented about 4-fold the levels of expression of all forms of bFGF at 48 hours (compare lane 7 with lane 6), but were unchanged at 24 hours (compare lane 5 with lane 4). Little or no bFGF expression, however, could be detected in explants cultured in DM alone (lane 8) or in DM+TPA (lane 9). Lanes 1-3 represent serial dilutions of the 18×10^3 M_r rh-bFGF used as standards. (B) Basic FGF expression in concentrated (about 100-fold) conditioned medium from the same cultures shown in A. Media were collected and applied to heparin-Affi-gel columns, eluted with 2.0 M NaCl, then concentrated and dialyzed as described in Materials and Methods. Note the lack of detectable levels of any of the bFGF isoforms in the conditioned medium from these same cultures. The amount of protein added to these lanes has been adjusted to represent the same numbers of cells as that shown in A. Again, lanes 1-3 represent serial dilutions of the 18×10^3 M_r rh-bFGF used as standards.

To confirm that the antisense oligonucleotides inhibited bFGF protein expression, cell lysates from TPA-treated peripheral nerve cultures were assayed for bFGF using protein immunoblotting methods, as described above. Fig. 3C shows that the antisense oligonucleotides significantly reduced TPA-induced bFGF expression in these cultures (compare lane 4 with 5 and lane 6 with 7). Densitometric analyses of such blots indicate that AS1 blocked the appearance of all TPA-induced isoforms of bFGF protein after 24 hours, and inhibited the appearance of these isoforms by greater than 80% after 48 hours. Together, these data indicate that the antisense oligonucleotides used in this study inhibited bFGF expression in peripheral nerve explant cultures and that this expression is necessary for the TPA-induced transdifferentiation into melanocytes.

The different isoforms of bFGF have been reported to be differentially localized within the nucleus or cytoplasm (Renko et al., 1990; Florkiewicz et al., 1991). To characterize the intracellular location of bFGF-immunoreactivity in Schwann cell precursors and to determine whether antisense oligonucleotide-treatment influenced this localization, peripheral nerve explant cultures were grown for 2 days in CM in the presence or absence of TPA and AS1, and then fixed and stained for bFGF-immunoreactivity. Fig. 4 shows that cultures treated with TPA showed significantly higher overall levels of bFGF immunoreactivity than untreated cultures (compare panels B and A), and that this immunoreactivity could be detected in the cytoplasm and, at slightly higher levels, in the nucleus. Cultures treated with both TPA and AS1 oligonucleotides displayed less overall bFGF-immunoreactivity (compare panels D and B), although this immunoreactivity was still present in both the nucleus and cytoplasm. In contrast, bFGF immunoreactivity was very low in negative controls, including TPA-treated cultures incubated without primary antibody (data not shown) and cultures incubated with primary antibody pre-absorbed with rh-bFGF (Fig. 4C).

Effects of bFGF-neutralizing antibodies and $InsP_6$ on TPA-induced transdifferentiation

Since the protein immunoblotting studies described above failed to show detectable levels of bFGF released into the

conditioned medium, it is unclear whether bFGF acts intracellularly or whether a small, but biologically important, fraction of bFGF is released and acts extracellularly. To distinguish between these possibilities, we cultured peripheral nerve explants in the presence or absence of TPA with either the DE6 monoclonal antibody, which blocks the effects of exogenously added human and quail bFGF (Reilly et al., 1989; Stocker et al., 1991), or inositol hexakisphosphate ($InsP_6$), which blocks the binding of bFGF to its extracellular receptors (Morrison, unpublished observations). Fig. 5A represents the time course of pigmentation in a representative experiment; Fig. 5B is the statistical analysis after 10 days of culture from four such experiments. These results indicate that both DE6 and $InsP_6$ completely inhibited the bFGF-induced pigmentation in peripheral nerve explants, but that neither of these agents had any effect on TPA-induced pigmentation.

A number of studies have shown that the bFGF secreted by some cells is deposited in the extracellular matrix (for review see Rifkin and Moscatelli, 1989), where agents such as neutralizing antibodies and $InsP_6$ may not be able to act. The extracellular matrix produced by cornea endothelial cells, for example, contains sufficient bFGF to promote proliferation of vascular endothelilal cells and differentiation of PC12 cells (Rogelj et al., 1989). To determine if the extracellular matrix of TPA-treated peripheral nerve cells contains biologically active bFGF that can promote Schwann cell precursor transdifferentiation, multiple peripheral nerve explants were cultured in the presence of 1.0 μM TPA for 2 days, at which time cells were observed to be confluent. Cells were then lifted from the plates by treating them with 0.1 mM EDTA in PBS (pH 7.3) for 15 minutes at room temperature. Plates were then washed with HBSS and fresh E7 peripheral nerves were cultured in CM alone on the remaining extracellular matrix. While 100% (12/12) of the control explants grown on these plates in the presence of 1.0 μM TPA produced pigment cells after 10 days, none of the cells (0/12) grown on these plates in CM alone became melanocytes. These data suggest that the bFGF expressed by Schwann cell precursors is not secreted and that at least some bFGF acts intracellularly in these cells.

Effects of bFGF-antisense oligonucleotides on bFGF-induced transdifferentiation

Although we show here that intracellular bFGF expression is necessary for the transdifferentiation of peripheral nerve cells, we had previously found that exogenous bFGF also induced melanogenesis in these cultures (Stocker et al., 1991). One possible explanation for this is that extracellular bFGF may itself induce intracellular bFGF expression (Weich et al., 1991). To test this possibility, peripheral nerve explants were grown in the presence of either bFGF or TPA plus or minus the bFGF antisense oligonucleotide AS1 and

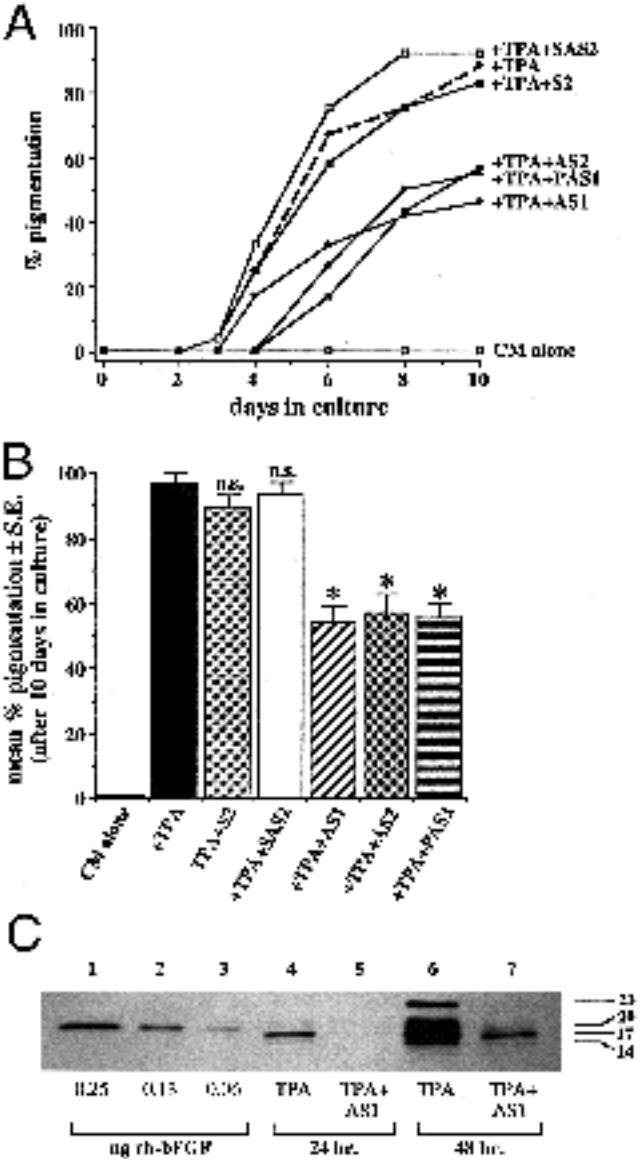

Fig. 3. Effects of bFGF-antisense oligonucleotides on TPA-induced pigmentation and bFGF expression in peripheral nerve explant cultures. Explants were grown for various periods of time in CM supplemented with 1.0 μM TPA in the presence or absence of 50 μM bFGF oligonucleotides (antisense oligonucleotides 'AS1' or 'AS2', scrambled antisense oligonucleotide 'SAS2', sense oligonucleotide 'S2') or 10 μM of phosphorothioate-modified bFGF antisense oligonucleotide ('PAS1'). Cultures were then either scored microscopically for the presence of pigment cells or assayed for bFGF expression using protein immunoblot analysis. (A) Time-course of pigmentation in one representative experiment. Note that the antisense and modified antisense oligonucleotides delayed the initial onset of TPA-induced pigmentation and reduced the final levels of pigmentation, whereas the sense and scrambled antisense oligonucleotides had no effects in this regard. (B) Means (± standard errors of the mean) of percent pigmentation after 10 days of culture from four separate experiments. Note that there was no statistically significant difference in pigmentation between cultures treated with TPA alone and cultures treated with either TPA+S2 or TPA+SAS2. All three antisense oligonucleotides, however, significantly inhibited TPA-induced pigmentation to a similar degree. (C) Protein immunoblot analysis of bFGF expression in peripheral nerve explant cell lysates treated with either TPA alone (lanes 4 and 6) or TPA+AS1 (lanes 5 and 7). Equal amounts of cellular protein (20 μg) were added to each of lanes 4-7. Note that AS1 blocked bFGF expression after 24 hours, and continued to inhibit up to 80% of bFGF expression after 48 hours. Lanes 1-3 represent serial dilutions of the 18×10^3 M_r rh-bFGF used as controls. n.s.=not significant; *P<0.05.

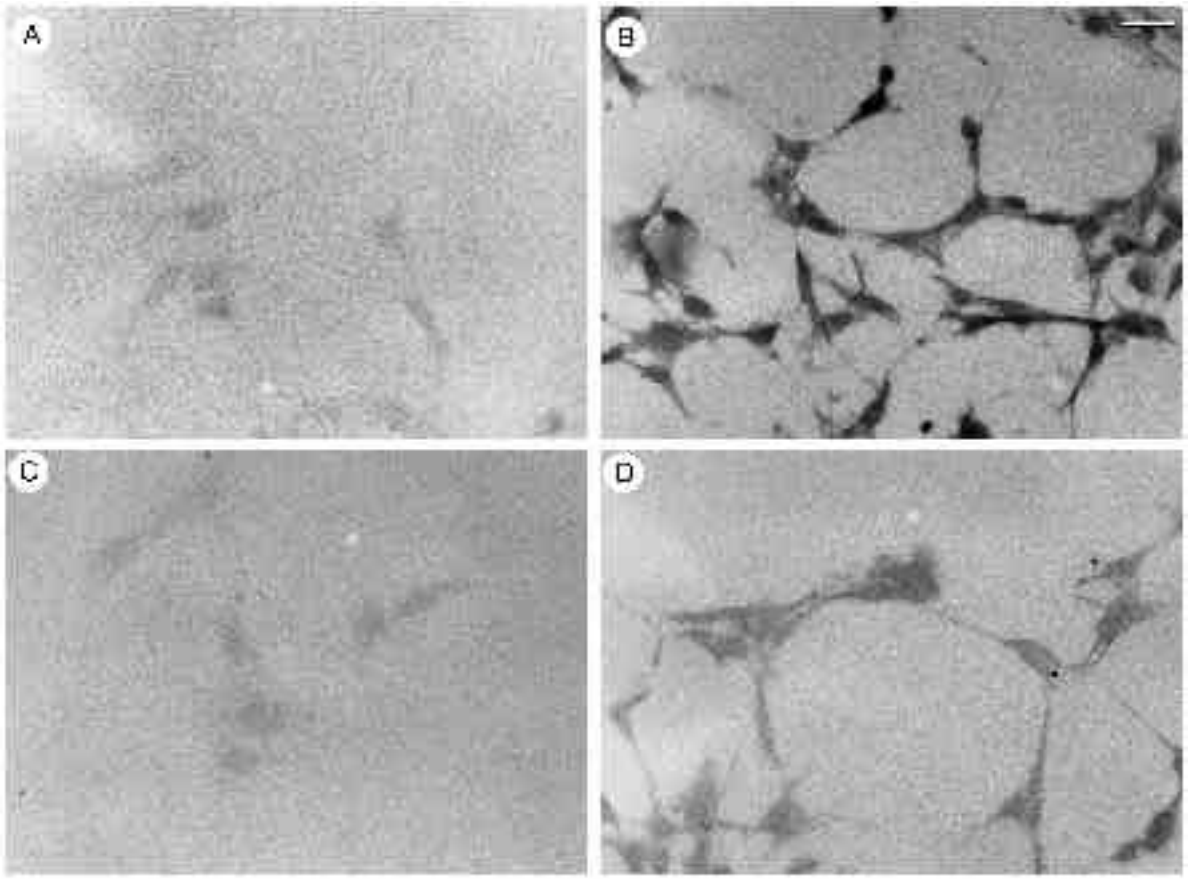

Fig. 4. Effects of bFGF antisense oligonucleotides on the immunocytochemical localization of bFGF in cultured peripheral nerve explants. Peripheral nerves from stage 32-33 quail embryos were cultured for 2 days in either CM alone (A), CM+1.0 μM TPA (B,C), or CM+TPA+50 μM AS1 (D). Cultures were then fixed and stained for bFGF immunoreactivity using a 1:150 dilution of monoclonal anti-bFGF antibody DE6, and then examined and photographed under bright-field photomicroscopy. (A) Basic FGF immunostaining in cells cultured in CM alone. Note the low level of bFGF immunoreactivity in the cell nuclei and cytoplasm. (B) Basic FGF immunostaining in cells cultured in CM+TPA. Note the presence of bFGF immunoreactivity in cell nuclei and a slightly lower levels in the cytoplasm. (C) Negative control for antibody specificity. These cultures were grown in CM+TPA, but were analyzed using DE6 preabsorbed with an excess of rh-bFGF. Note that staining was reduced to background levels. (D) Basic FGF immunostaining in cells cultured in CM+TPA+AS1. Note that the bFGF antisense oligonucleotides reduced the overall levels of TPA-augmented bFGF expression, but that immunostaining could still be detected in both cell nuclei and cytoplasm. The bar in the upper right corner represents 30 μm.

then examined at the end of 10 days for the presence of pigmented cells. Fig. 6 shows that AS1 significantly blocked TPA-induced pigmentation, but had no significant effects on bFGF-induced pigmentation. This experiment was performed three times with qualitatively similar results. These data suggest, therefore, that induction of intracellular bFGF by exogenously added bFGF does not play a significant role in the transdifferentiation of Schwann cell precursors into melanocytes.

Effects of bFGF and TPA on BrdU incorporation

In previous work, we found that TPA caused a greater proportion of peripheral nerve explant cultures to become pigmented than exogenously added bFGF (Stocker et al., 1991). Extracellular bFGF may, therefore, have other effects on peripheral nerve cells, such as acting as a mitogen. This notion is supported by reports that TPA and/or bFGF are mitogens for rodent Schwann cells (Krikorian et al., 1982; Eccleston et al., 1987; Ratner et al., 1988; Davis and Stroobant, 1990; Schubert, 1992), human melanocytes (Eisinger and Marko, 1981; Halaban et al., 1987) and avian NC cells (Sieber-Blum and Sieber, 1981). To determine whether TPA and bFGF were mitogenic for embryonic quail peripheral nerve cells, explant cultures were grown for 47 hours in the presence or absence of either 1.0 μM TPA or 20 ng/ml rh-bFGF, and then these cultures were assayed for mitogenesis using a BrdU-incorporation assay. Fig. 7 shows that TPA caused a statistically significant increase in the BrdU-labelling index (approximately 40%; n=6; P<0.01). Exogenously added bFGF, however, caused a significantly greater increase in the labeling index (approximately 110%; n=6; P<0.005). Cultures treated with both TPA and bFGF did not contain significantly more $BrdU^+$ cells than cultures treated with either agent alone. These experiments were performed three times with qualitatively similar results.

DISCUSSION

A fundamental question in developmental biology concerns the cellular mechanisms by which multipotent cells become committed to particular cell fates. Clues about these underlying mechanisms may be inferred from observations of various experimentally induced transdifferentiation events, in which embryonic cells transform from one phenotype to another (Aloe and Levi-Montalcini, 1979; Okada, 1980; Doupe et al., 1985; Pittack et al., 1991). Such transdifferentiation events may reveal aspects of both the lineage relationship between the two cell types, as well as clues concerning molecular mechanisms responsible for the determination of their phenotype.

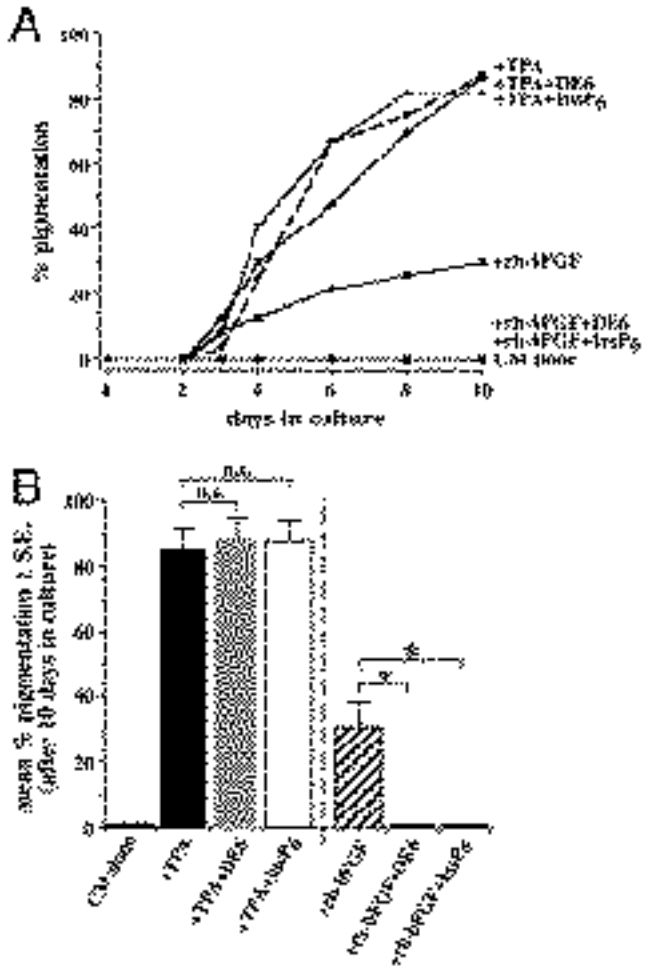

Fig. 5. Effects of bFGF-neutralizing antibodies and inositol hexakisphosphate ($InsP_6$) on TPA-induced melanogenesis. Peripheral nerve explants from stage 32-33 quail embryos were grown for various periods of time in CM supplemented with either 1.0 μM TPA or 20 ng/ml rh-bFGF in the presence or absence of a 1:250 dilution of monoclonal anti-bFGF antibody DE6 or 25 μM $InsP_6$. Cultures were then scored for the presence of pigment cells. (A) Time-course of pigmentation in one representative experiment. Note that neither DE6 nor $InsP_6$ affected the onset or extent of pigmentation as compared to cultures treated with TPA alone (broken line), but that both of these agents blocked completely the rh-bFGF-induced pigmentation (compare with dotted line). (B) Means (± standard errors of the mean) of percent pigmented cultures after 10 days from 4 separate experiments. Note the lack of statistically significant differences in pigmentation between cultures treated with TPA alone and cultures treated with TPA+DE6 or TPA+$InsP_6$. Pigmentation induced by rh-bFGF, however, was totally blocked by both of these agents. n.s.=not significant; *P<0.05.

Expression of bFGF is necessary for the transdifferentiation of Schwann cell precursors into melanocytes

In previous work, we found that both bFGF and TPA induced the adventitious appearance of pigmented cells in cultured embryonic quail peripheral nerve explants, presumably due to the transdifferentiation of Schwann cell precursors into melanocytes (Ciment et al., 1986; Sears and Ciment, 1988; Stocker et al., 1991). We report here that the cells undergoing TPA-induced pigmentation in these cultures express the P_0 protein, suggesting that they are part of the early Schwann cell lineage. That is, since most of the peripheral nerve cell cultures contain only P_0^+ cells, and since most of the TPA-treated cultures contain pigmented cells, it seems likely that at least some of the pigmented cells arose from P_0^+ antecedents. The possibility that a few pigmented cells also arose from the small numbers of P_0^- cells in these cultures cannot, however, be ruled out.

We also found that bFGF expression by these cells is necessary for this transdifferentiation. This conclusion is based on the observations that (i) TPA augmented the expression of the various bFGF protein isoforms in peripheral nerve cultures and (ii) interference with bFGF expression by treatment with specific antisense oligonucleotides significantly inhibited the extent of TPA-induced

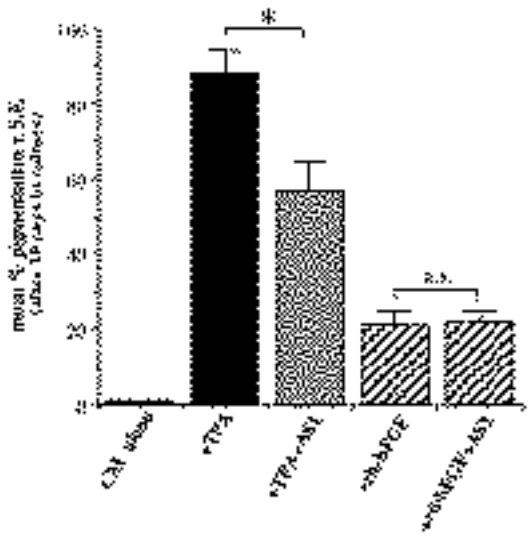

Fig. 6. Effects of bFGF antisense oligonucleotides on melanogenesis induced by exogenous bFGF. Peripheral nerve explants from stage 32-33 quail embryos were cultured for 10 days in CM supplemented with either 1.0 μM TPA or 20 ng/ml rh-bFGF in the presence and absence of 50 μM AS1, and then assayed for pigment cells. Note that AS1 significantly inhibited TPA-induced pigmentation, but had no significant effect on rh-bFGF-induced pigmentation. Data are plotted as the means ± s.e.m. for 3 experiments. n.s.=not significant; *P<0.05.

melanogenesis. Although treatment with the bFGF antisense oligonucleotides produced only a partial inhibition of this melanogenesis, there are reasons to believe that these effects were specific and mediated by inhibition of bFGF protein expression. First, two different antisense oligonucleotides, corresponding to non-overlapping regions of the mRNA transcript, produced similar inhibition of melanogenesis in peripheral nerve cells, whereas the corresponding sense oligonucleotides and scrambled antisense oligonucleotides had no significant effects in this regard. Second, antisense oligonucleotides transiently inhibited bFGF protein expression in these cultures, as shown by both protein immunoblotting and immunocytochemical methods. The partial inhibition of pigmentation may have been due to the rate at which the antisense oligonucleotides were taken up into cells, or their rate of degradation (Toulmé and Hélène, 1988). Phosphorothioate-modified oligonucleotides, which are presumably less susceptible to nuclease degradation (Marcus-Sekura et al., 1987), were found, however, to be no more effective at inhibiting TPA-induced transdifferentiation than non-modified oligonucleotides. The constitutive expression of bFGF protein, which we observed in non-TPA-treated Schwann cell precursors, may explain why some transdifferentiation occurred in these cultures, since antisense oligonucleotides would not affect the levels of proteins already synthesized. This notion is supported by the observations that bFGF protein is highly resistant to degradation, especially in the presence of heparin sulfate proteoglycans (Gospodarowicz and Cheng, 1986). It is possible, therefore, that some of the Schwann cell precursors in peripheral nerve explants may have contained sufficient levels of bFGF at the time of culture to facilitate their TPA-induced transdifferentiation into melanocytes.

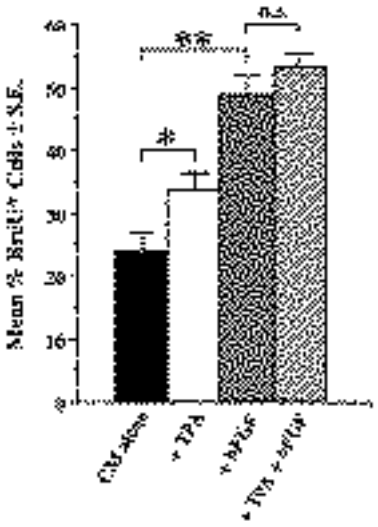

Fig. 7. Mitogenic effects of TPA and exogenous bFGF on cultured peripheral nerve explants. Individual peripheral nerve explants from stage 32-33 quail embryos were cultured for 47 hours in CM supplemented with either 1.0 μM TPA, 20 ng/ml rh-bFGF, or both TPA and rh-bFGF. The cultures were then labelled for 1 hour with bromodeoxyuridine (BrdU) and then fixed. Cells incorporating BrdU were then identified using a kit, as described in Materials and Methods. Note that TPA only weakly stimulated mitogenesis in these cultures (34%), while rh-bFGF strongly stimulated mitogenesis (48%). There was no significant difference in the BrdU labeling index between cultures treated with bFGF alone and cultures treated with bFGF+TPA. Each datum point is plotted as the mean ± s.e.m. of six separate cultures. n.s.=not significant; **P*<0.01; ***P*<0.005.

Interestingly, we found no bFGF expression in TPA-treated PN explants cultured in a serum-free medium. There was also no basal bFGF expression in these cultures, indicating that some component of serum induces bFGF expression. These observations are in agreement with previous studies that have shown that serum can induce bFGF mRNA expression (Murphy et al., 1988b). Furthermore, we previously showed that TPA did not cause melanogenesis in DRG explants cultured in the absence of serum, unless exogenous bFGF was also present (Stocker et al., 1991). Serum has been shown to cause the translocation of intracellular bFGF from the cytosol to the nucleus (Yamamoto et al., 1991), which could somehow prime early Schwann cells to respond to TPA. Taken together, these findings indicate that bFGF is necessary, but not sufficient, for the transdifferentiation of Schwann cell precursors into melanocytes.

Only a subpopulation of P_0^+ cells underwent pigmentation in response to TPA in the presence of serum, even though all of these cells expressed intracellular bFGF. These data indicate that there was some heterogeneity among these Schwann cell precursors. Although the nature of this heterogeneity is not clear, there are at least two possible explanations. First, there may be a differential responsiveness to these agents depending on the phase of the cell cycle. Schwann cells have relatively long cell cycles (e.g., Eccleston et al., 1987) and it is possible that the phase in which initial exposure to TPA or bFGF occurs is a critical factor in determining whether the cell undergoes transdifferentiation. Alternatively, these cultures may consist of Schwann cell precursors at different stages of their commitment, with differential responsiveness to exogenous TPA and bFGF. This notion is supported by previous observations that peripheral nerve explant cultures established from progressively older quail embryos demonstrate decreasing levels of TPA-induced pigmentation (Ciment et al., 1986). These data would suggest, therefore, that the developmental period in which TPA or bFGF can induce transdifferentiation of Schwann cell precursors into melanocytes is rather limited.

The three protein isoforms of bFGF found in cell lysates of quail peripheral nerve cultures were found to be similar in their sizes and relative abundances to those previously found in homogenates of early avian embryos (Sherman et al., 1991). One isoform had a relative molecular mass of 20 $\times 10^3$ and was weakly expressed, while two isoforms had relative molecular masses of 17 and 23×10^3 and were strongly expressed in these embryonic quail peripheral nerve cells. The M_rs, tissue distribution and number of these isoforms are different in various animal species (Brigstock et al., 1990; Sherman et al., 1991; Giordano et al., 1992). In humans, for example, there are four forms of the human bFGF protein with M_rs of 18, 22, 22.5 and 24 $\times 10^3$, all derived from a common mRNA. Translation of the 18×10^3 M_r form initiates from an AUG codon, while the higher

relative molecular mass forms initiate from CUG codons 5′ of the AUG codon (Florkiewicz and Sommer, 1989; Prats et al., 1989). Although the function of these amino terminal extended forms of bFGF remains unclear, recent studies indicate that the higher relative molecular mass forms are localized exclusively in the nucleus (Bugler et al., 1991; Florkeiwicz et al., 1991) and that the selective expression of these isoforms may have diverse effects on various cellular behaviors (Couderc et al., 1991; Quarto et al., 1991a). Since all three isoforms were inhibited by the antisense oligonucleotides in these peripheral nerve cultures, however, it is not clear whether all or one of these isoforms were responsible for TPA-induced transdifferentiation of peripheral nerve cells. Therefore, it is conceivable that the selective expression of one of these bFGF isoforms in Schwann cell precursors of avian embryos may have been sufficient to promote their transdifferentiation into melanocytes.

It is not entirely clear how bFGF expression is regulated by TPA-treatment in avian and other species. TPA is known to bind to protein kinase C (PKC) and cause its translocation to the plasma membrane, resulting in its increased rate of degradation (Nishizuka, 1986). In previous work, we found that the time course of TPA-induced pigmentation was consistent with that of PKC down-regulation in avian NC-derived cells (Hess et al., 1988), suggesting that the loss of PKC activities somehow participates in the transdifferentiation process. The human bFGF gene, moreover, contains a potential TPA-responsive element in its promoter region (Shibata et al., 1991), and its presence within the avian bFGF gene could explain how TPA could augment bFGF expression in these Schwann cell precursors. This notion is supported by the observations that TPA, at concentrations that both activate and down-regulate PKC, induces bFGF mRNA expression in a wide variety of other cell types (Murphy et al., 1988a; Bikfalvi et al., 1990; Weich et al., 1991; Lowe et al., 1992).

Basic FGF acts in Schwann cell precursors via an intracrine mechanism

These studies also indicate that the bFGF expressed by Schwann cell precursors is not secreted and is likely not to interact with extracellular receptors. Neither bFGF-neutralizing antibodies nor $InsP_6$, for example, affected the TPA-induced transdifferentiation of these cells into melanocytes, even though the effects of exogenously added bFGF were totally blocked by these agents. It is unlikely that bFGF was released into the extracellular matrix of these cultured Schwann cell precursors and was inaccessible to blocking agents, since the extracellular matrix from TPA-treated peripheral nerve explants was not capable of promoting melanogenesis and did not appear to augment TPA-induced melanogenesis. Furthermore, bFGF was not detected in the concentrated conditioned medium of cultured peripheral nerve explants and immunocytochemistry revealed high levels of bFGF expression in both the cytosol and nucleus of these TPA-treated Schwann cell precursors. It is likely, therefore, that bFGF remains within these cells, where it acts directly as an intracellular signal.

While secretion has conventionally been considered a prerequisite of growth factor activity, these and other data support the notion that bFGF can act by means of an intracrine mechanism (Logan, 1990). Several studies have shown, for example, that bFGF is localized within the nucleus and nucleolus (Kardami and Fandrich, 1989; Joseph-Silverstein et al., 1989; Renko et al., 1990; Kalcheim and Neufeld, 1990; Tessler and Neufeld, 1990; Yamamoto et al., 1991; Woodward et al., 1992), which are not conventional locations for secreted proteins, in addition to its localization within the cytoplasm. Indeed, the amino terminal extension of the higher molecular weight forms of bFGF contains a nuclear localization sequence that has been shown to cause translocation of heterologous gene products into nuclei (Bugler et al., 1991; Quarto et al., 1991b; Florkiewicz et al., 1991). Moreover, various cells transfected with bFGF-expression constructs have demonstrated increased growth characteristics, even though little or no bFGF was detected in their conditioned media (Neufeld et al., 1988; Halaban et al., 1988). In these experiments, the transformed state of these transfected cells was not affected by bFGF-neutralizing antibodies, indicating that released bFGF was not required for proliferation.

Additional support for the notion that bFGF can act via an intracrine pathway comes from studies suggesting that intracellular bFGF directly influences gene transcription. In isolated nuclei from bovine aortic endothelial cells, for example, bFGF was shown to stimulate transcription of ribosomal genes and to increase RNA polymerase I activity (Bouche et al., 1989). In nuclear extracts from Ehrlich ascites tumor cells, bFGF influenced transcription of genes encoding isozymes of phosphoglycerate kinase, which are involved in a transcriptional switch during spermatogenesis from somatic-type to sperm-specific forms of the enzyme (Nakanishi et al., 1992). Furthermore, bFGF, which has been implicated in amphibian mesoderm induction (Kimelman and Kirschner, 1987), was recently shown to translocate from the cytosol of cleavage and blastula stage cells of *Xenopus* embryos to cell nuclei during mid-blastula transition (Shiurba et al., 1991). These data suggest that some of the biological activities attributed to bFGF may be mediated by nuclear bFGF binding proteins or by the direct interaction of bFGF with DNA.

Intracellular and extracellular bFGF may have different effects on mitogenesis of Schwann cell precursors

Previous studies have shown that exogenous bFGF can act as a Schwann cell mitogen in various mammalian species, especially when administered with agents that increase intracellular dibutyryl cyclic AMP (Krikorian et al., 1982; Eccleston et al., 1987; Ratner et al., 1988; Davis and Stroobant, 1990) or in the presence of transforming growth factor β (TGF-β) (Schubert, 1992). In the present study, we found that exogenous bFGF can act as a strong mitogen for embryonic quail Schwann cell precursors in the presence of serum. TPA, however, had only weak mitogenic activity in these cultures and did not influence the mitogenic activity of bFGF. These observations are consistent with the notion that the effects of intracellular bFGF on Schwann cell precursors are distinct from the effects of extracellular bFGF, which presumably interacts with cell surface receptors. There are four known human FGF receptor genes (FGFR1-FGFR4), each encoding distinct high affinity tyrosine kinase

receptors (Houssaint et al., 1990; Johnson et al., 1990; Keegan et al., 1991; Partanen et al., 1991). Multiple proteins are derived from each of these genes by alternative mRNA splicing and each protein is thought to have different affinities for the various members of the FGF family (Johnson et al., 1991; Eisemann et al., 1991; Miki et al., 1991). It is likely, therefore, that the number and types of receptors expressed by NC-derived cells determine how these cells will respond to extracellular bFGF and FGF family members. Although the localization and specificities of these FGF receptors are still largely unknown, some migrating NC cells have been shown to express FGFR1 mRNA transiently (Heuer et al., 1990).

Our finding that both exogenously added bFGF and intracellular bFGF induce the transdifferentiation of Schwann cell precursors into melanocytes raises the possibility that exogenous bFGF may have induced its own expression (Weich et al., 1991). Since we found that bFGF antisense oligonucleotides had no significant effects on bFGF-induced pigmentation in these cultures, however, this possibility is unlikely. On the contrary, it is possible that exogenous bFGF might be internalized by Schwann cell precursors to act intracellularly. Various polypeptide growth factors, including bFGF, are known to be translocated into the nucleus after cell surface binding and internalization (Rakowicz-Szulczynska et al., 1986; Bouche et al., 1987; Baldin et al., 1990; Hawker and Granger, 1992). In one study, bFGF was found to be internalized both via high affinity binding sites, presumably one of the FGF tyrosine kinase receptors, and via low affinity heparin-binding proteoglycans (Gannoun-Zaki et al., 1991). Since heparin protects bFGF from proteolytic degradation (Gospodarowicz and Cheng, 1986), it seems likely that some bFGF-proteoglycan complexes may have remained intact inside cells (Hawker and Granger, 1992). These observations may explain some of the dose-dependent effects of bFGF. In a recent study, for example, BHK-21 cells producing low amounts of bFGF were cocultured with NC cells and found to have a moderate effect on neurogenesis, but no effect on the appearance of non-neuronal cells (Brill et al., 1992). BHK-21 cells releasing high levels of bFGF, however, caused an increase in both neuronal and non-neuronal phenotypes in cocultured NC cells. This may suggest that Schwann cell progenitors that encounter low extracellular concentrations of bFGF in the embryo would proliferate, while cells encountering higher concentrations of bFGF, or agents that elevate intracellular bFGF, would differentiate into melanocytes.

Endogenous growth factors may influence the fate of the melanocyte/Schwann cell progenitor

The notion of the NC-derived melanocyte/Schwann cell progenitor is supported by various pieces of evidence. In previous work, we found that cells with latent melanogenic potential existed transiently during development in peripheral nerves and other NC-derivatives known to contain Schwann cell precursors. These cells have the ability to migrate normally along NC migratory pathways following grafting into host embryos in vivo, where they give rise to melanocytes and Schwann cells, but not various other NC-derived phenotypes (Sears and Ciment, 1988). Further support for the notion of a melanocyte/Schwann cell progenitor comes from various clonal analysis studies performed in culture and in vivo in which mixed clones of melanocytes and Schwann cells were observed (Dupin et al., 1990; Bronner-Fraser and Fraser, 1989). Finally, various human pathologies have been described in which Schwann-like cells seem to undergo a transdifferentiation into melanocytes, but not to other NC-derivatives (El-Labban, 1988). In the human genetic disease von Recklinghausen's neurofibromatosis, for example, benign tumors of Schwann-like cells often contain pigment cells or are found to underly spots of hyperpigmentation in the skin (Rubenstein, 1986).

Our observations suggest that extracellular bFGF is not the instructive signalling molecule directing the fate of these putative melanocyte/Schwann cell progenitors to become committed to melanogenesis. One candidate molecule for this function, however, might be Steel factor (SLF), which has structural homologies to various growth factors (Huang et al.,, 1990). Mutations of the gene encoding SLF or its cell surface receptor, the tyrosine kinase c-kit, disrupt a number of developmental processes in mice, including melanogenesis (Williams et al., 1992). It has recently been shown, moreover, that combinations of SLF and TPA strongly promote melanogenesis in cultures of murine NC cells (Murphy et al., 1992), and that neutralizing antibodies against the c-*kit* receptor inhibit melanogenesis in vivo (Nishikawa et al., 1991). A second potential factor involved in cell fate decisions of melanocyte/Schwann cell progenitors might be the unknown factor(s) found in serum-containing medium that permit bFGF or TPA to induce melanogenesis in peripheral nerve cultures (see also Stocker et al., 1991). This notion is supported by previous reports of unknown factors in serum that induce bFGF mRNA expression (Murphy et al., 1988b), or which cause the translocation of intracellular bFGF from the cytosol to the nucleus (Yamamoto et al., 1991).

In contrast, one candidate molecule that might be involved in promoting Schwann cell development in melanocyte/Schwann cell progenitors is TGF-β, which has been shown to be present in the NC migratory space (Jakolew et al., 1993). TGF-β inhibits both the TPA- and bFGF-induced appearance of melanocytes in embryonic quail dorsal root ganglia and peripheral nerve explants (Stocker et al., 1991), and inhibits melanogenesis in NC cultures (Rogers et al., 1992). TGF-β has also been found to act synergistically with bFGF to promote Schwann cell proliferation (Schubert, 1992), but does not influence differentiation. It remains to be seen, however, whether the effects of TGF-β are simply to neutralize the effects of melanogenic factors, or whether it induces the expression of Schwann cell traits in melanocyte/Schwann cell progenitors.

It seems likely that these and other extracellular signaling molecules play a role in the commitment of NC-derived cells to the melanocyte or Schwann cell lineages. Determining whether intracellular bFGF mediates the effects of these other signaling molecules may provide further insights into how these events eventually regulate the constellation of phenotype-specific gene products that define the melanocyte or Schwann cell phenotype.

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