INTRODUCTION

Although the developmental regulation of myelin formation by Schwann cells has been intensively studied, many questions still remain unanswered. Thus the molecular nature of the molecular signals that regulate and control myelin formation in Schwann cells is not well understood.

Our previous experiments on rat Schwann cells in vitro, using serum-free defined medium, showed that a myelin-related protein phenotype could be induced in early postnatal Schwann cells in culture by elevation of intracellular cyclic AMP levels in the absence of growth factors, conditions under which the cells are not dividing. Cells with this phenotype expressed the major myelin glycoprotein Po and expression of p75 NGF receptor, N-CAM, GFAP and A5E3 proteins was down-regulated. These changes are all characteristics associated with myelination in vivo. In contrast, when cyclic AMP levels were elevated in the presence of serum, suppression of cyclic AMP-induced differentiation resulted and DNA synthesis was induced.

In this paper, we have used this model system and extended our analysis to explore the relationship between defined growth factors and suppression of myelination. We have used pure recombinant growth factors normally present in peripheral nerves, i.e. FGF1 and FGF2 and TGFβ1, TGFβ2, and TGFβ3 and shown that, like serum, they can strongly suppress the forskolin-mediated induction of the Po gene, both at the level of mRNA and protein synthesis. For both growth factor families, the suppression of Po gene expression is dose-dependent and takes place in serum-starved cells that are mitotically quiescent.

In the case of FGF2, however, even more complete suppression is obtained when the cells are simultaneously allowed to enter the cell cycle by inclusion of high concentrations of insulin in the culture medium. The present results raise the possibility that, in addition to the positive axonal signals that are usually envisaged to control the onset of myelination, growth factors present in the nerve may exert negative regulatory signals during development and thus help control the time of onset and the rate of myelination in peripheral nerves.

Key words: DNA synthesis, in situ hybridisation, forskolin, differentiation, cyclic AMP, Schwann cell, Po gene, TGFβ1, TGFβ2, TGFβ3

SUMMARY

Negative regulation of the Po gene in Schwann cells: suppression of Po mRNA and protein induction in cultured Schwann cells by FGF2 and TGFβ1, TGFβ2 and TGFβ3

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INTRODUCTION

During the development of peripheral nerves, Schwann cells are induced to form myelin sheaths round the larger axons. This process involves a complex series of events and the nature of the molecular signals that regulate and control myelin formation in Schwann cells is not well understood.

High levels of expression of myelin proteins and associated down-regulation of molecules characteristic of embryonic Schwann cells and mature non-myelin-forming Schwann cells, are only seen in vivo at the time that the myelin sheath can be seen morphologically (for review see Jessen and Mirsky, 1991). The major PNS protein Po has been particularly studied in this respect and it has served as an indicator of myelin-related differentiation in several studies both in vivo and in vitro (Brockes et al., 1980; Mirsky et al., 1980; Lemke and Axel, 1985; Trapp et al., 1988; Stahl et al., 1990). It has been reported that Po mRNA is detectable at embryo day (E) 18, which is 2-3 days before myelin can be seen morphologically in the rat sciatic nerve (Kamholz et al., 1992) and we have observed Po mRNA expression in this nerve at E 17 (M.-J Lee, M. Dent, K. R. Jessen and R. Mirsky, unpublished observations). Low levels of Po protein located in the Golgi apparatus of some cells have also been reported prior to myelination (Trapp et al., 1981) although high levels of protein expression coincide with the onset of myelination (Mirsky et al., 1980;
Brockes et al., 1980). Neither myelin nor high levels of \( \text{P}_0 \) protein are detectable in association with dividing Schwann cells (Friede and Samorajski, 1968; Brown and Ashbury, 1981; Martin and Webster, 1973; Webster et al., 1973; Stewart et al., 1993). Studies on DNA synthesis in Schwann cells in vivo show that high levels of DNA synthesis are seen throughout embryonic life with a peak at E 19, declining rapidly thereafter (Stewart et al., 1993).

Our previous experiments on rat Schwann cells in vitro, using serum-free defined medium, show that a myelin-related protein phenotype can be induced in early postnatal Schwann cells in culture by elevation of intracellular cyclic AMP (cAMP) levels in the absence of growth factors, conditions under which the cells are not dividing (Morgan et al., 1991). Thus cells are induced to express immunocytochemically detectable levels of \( \text{P}_0 \) protein and in those cells expressing the highest levels of \( \text{P}_0 \), p75 NGF receptor (NGFR), N-CAM, GFAP and A5E3 protein are all down-regulated, characteristic changes associated with myelination in vivo (Jessen et al., 1984, 1987, 1990; Morgan et al., 1991).

We found also that inclusion of serum or impure preparations of glial growth factor in this type of experiment resulted in suppression of cAMP-induced myelin differentiation, while an early Schwann cell differentiation step also induced by cAMP in vitro, i.e. 04 appearance, was unaffected. From experiments in which Schwann cell proliferation was regulated by plating density, we further concluded that the suppressive effect of serum depended on the proliferation induced by cAMP elevation in the presence of serum, and that the inhibitory pathway was therefore initiated down-stream of the mitogenic response (Mirskey et al., 1990; Morgan et al., 1991).

In contrast to our studies, most other experiments on cultured purified Schwann cells have not used serum-free medium, but have used instead medium containing either serum or plasma to which growth factors or cAMP elevating drugs such as forskolin are then added. It is clear that, under these conditions, galactocerebroside, \( \text{P}_0 \) and myelin basic protein mRNA and \( \text{P}_0 \) protein levels can be significantly elevated in response to cAMP analogues or forskolin (Sobue and Pleasure, 1984; Sobue et al., 1986; Lemke and Chao, 1988; Monuki et al., 1989; Mews and Meyer, 1993). Three factors make it difficult to compare the results directly with ours, particularly with respect to the existence of a serum-dependent inhibition of cAMP-induced \( \text{P}_0 \) gene expression that might be related to DNA synthesis. Firstly, sera differ considerably in growth factor content; secondly, Schwann cells show strong density-dependent inhibition of proliferation and, thirdly, DNA synthesis was not measured in these experiments.

The existence of a negative component in the set of cell-extrinsic signals that control myelin synthesis by Schwann cells would have important implications for our understanding of how the timing and speed of myelination is regulated during nerve development. It would also throw light on pathological failures of myelination and myelin maintenance by Schwann cells.

In the present paper, we have therefore analysed this issue further. We have used pure recombinant growth factors normally present in peripheral nerves, i.e. FGF1 and FGF2 (Kalcheim and Neufeld, 1990) and TGF\( \beta \), TGF\( \beta \)2 and TGF\( \beta \)3 (Flanders et al., 1991; Unsicker et al., 1991; Scherer et al., 1993), analysed their effect on the forskolin-mediated induction of the \( \text{P}_0 \) gene both at the level of \( \text{P}_0 \) mRNA and protein expression in highly purified Schwann cell cultures maintained in defined medium, and related the results to the presence or absence of DNA synthesis. In sum, our results with FGF1 and FGF2 confirm our previous findings in serum, in revealing an inhibitory component that depends on conditions that induce Schwann cells to enter the cell cycle, synthesize DNA and proliferate. More importantly however, using FGF1 or FGF2 plus lowered insulin concentrations or using TGF\( \beta1 \), TGF\( \beta2 \) or TGF\( \beta3 \), we also show the existence of a powerful inhibition of cAMP-induced \( \text{P}_0 \) gene expression that is independent of a mitogenic response, occurring under conditions that do not induce DNA synthesis. Thus myelin-formation may, like many other developmental processes, be driven by a complex mixture of positive and negative regulatory signals.

MATERIALS AND METHODS

Schwann cell culture

Sciatic nerves were dissected from postnatal rats aged 6 days old and desheathed. The tissue was dissociated essentially by the method of Brockes et al. (1979) and maintained in Dulbecco’s modified Eagles’ medium (DMEM) with added glutamine, insulin and 10% calf serum. Cysteine arabinoside (10-5 M) was added after 24 hours for 72 hours resulting in cultures that were 99% pure. On the fourth day, cells were plated onto coverslips coated with poly-L-lysine plus laminin as described previously (Morgan et al. 1991), at densities of between 5,000-20,000 cells/coverslip. Cells were left for 24 hours before further treatment. At this stage, cells were cultured in defined medium which consisted of a 1:1 mixture of DMEM and Ham’s F12 medium supplemented with BSA (Penetix) (30 mg ml-1), dexamethasone (38 ng ml-1), insulin (5 \( \mu \)g ml-1), transferrin (100 \( \mu \)g ml-1), triiodothyronine (10 ng ml-1) and selenium (160 ng ml-1), all purchased from Sigma Chemical Co. Note that the composition of this medium is incorrectly printed in a previous paper (Morgan et al., 1991). In one set of experiments, insulin was used at a concentration of 5 ng ml-1 (8.7x10-10 M).

The purity of our cultures, monitored as the percentage of total cells that are S100 positive at 4 days in vitro is 99±0.4%.

In all experiments involving cell counts, experiments were repeated a total of at least three times. In each experiment, two (mRNA experiments) or three (protein experiments) coverslips were used for each experimental point, and a minimum of 200 cells counted per coverslip. As a control for experiments involving northern blotting or immunoblotting BALB/c 3T3 fibroblasts were grown in 10% FCS and DMEM.

Cell culture in the presence of growth factors, forskolin and thymidine

Recombinant human FGF2 (basic FGF) (Prepro Biotechnology) was added to the cultures at concentrations varying from 0.001-50 ng ml-1 (0.059 pM-2.94 nM). Human recombinant FGF1 (acidic FGF) (a gift from M. Jaye von Rorer Biotechnology) was used at a concentration of 10 ng ml-1 (0.63 nM) in the presence of heparin (20 \( \mu \)g ml-1) (Sigma Chemical Co.). Human recombinant TGF\( \beta1 \) (Boehringer Mannheim), porcine TGF\( \beta2 \) and recombinant chicken TGF\( \beta3 \) (British Biotechnology) were used at concentrations varying from 0.001 to 1 ng ml-1 (0.04-40 pM). All growth factors were replaced every 24 hours. In some experiments, forskolin (4 \( \mu \)M) was added in the presence and absence of growth factors. A 10 mM stock in alcohol was diluted in medium. The forskolin was replaced in new medium every 24 hours, and the final concentration of ethanol in the medium was 0.04%. Experiments in which ethanol was used alone at
this concentration showed that it had no effect on the parameters being measured. Thymidine was used at concentrations from 10-300 μM (Sigma Chemical Co).

**Antibodies**

Mouse monoclonal antibody to bromodeoxyuridine (Brdu) Bu20a in the form of supernatant (Gratzner, 1982) was used diluted 1:50. Two different rabbit polyclonal P0 antibodies were used; the first, a gift from Dr B. D. Trapp, was used in immunoblots diluted 1:4,000 and the second, made in this laboratory essentially as described by Brocks et al. (1980), was used diluted 1:500 for immunocytochemistry. Mouse monoclonal antibody to the extracellular domain of P0, P07, was a gift from Dr J. J. Archelos (Archelos et al., 1993). It was used diluted 1:10,000 in western blots. Rabbit polyclonal antibodies to S100 protein (Dakopatts) were used diluted 1:1,000. Goat antimouse Ig conjugated to tetramethyl rhodamine (Cappel Organon Teknika Corp) was used diluted 1:200. Cross reactivity to rabbit Ig was removed by adsorption against rabbit Iggs. Donkey anti-rabbit Ig conjugated to biotin was used diluted 1:100 for immunocytochemistry and 1:1,000 for immunoblotting. Streptavidin-peroxidase conjugated (Amersham International plc) was used diluted 1:100. Non-specific binding to anti-rabbit Ig was removed by incubation overnight at 4°C with chopped adult rat nerve. Polyclonal sheep anti-digoxigenin conjugated to alkaline phosphatase (Boehringer Mannheim) was used diluted 1:2,500. Peroxidase-conjugated rabbit anti-mouse Ig (Dakopatts) was used diluted 1:1,000 in immunoblots.

**In situ hybridisation**

A digoxigenin-labelled cDNA probe was used to detect P0 mRNA in Schwann cell cultures, detecting hybridisation by alkaline phosphatase-linked immunohistochemistry. A cDNA (SN63c) encoding the entire P0 coding sequence (1.8 kb) subcloned into pGEM4, donated by Drs G. Lemke and I. Griffiths was used (Lemke and Axel, 1985; Griffiths et al., 1989). Digoxigenin-labelled probes were transcribed using the Boehringer SP6/T7 transcription kit and manufacturer’s instructions. Transcripts were hydrolysed to give an average probe length of 150 bases and used at a concentration of 2.5 ng μl⁻¹. Schwann cells on coverslips were fixed in paraformaldehyde and dehydrated in 70% ethanol. The cells were rehydrated, digested for 3.5 minutes in proteinase-K 2.5 μg ml⁻¹ at room temperature, refixed in paraformaldehyde and treated with 0.1 M triethanolamine acetate. After hybridisation at 37°C in 50% formamide for at least 4 hours, cells were digested in 4x SSC at 45°C for 10 minutes at room temperature, digested in RNase-A for 30 minutes at 37°C, washed in 2x SSC at 45°C for 30 minutes and 0.1x SSC at 55°C for 30 minutes. Cells were then blocked in 5% fat-free milk in PBS for 30 minutes, and incubated in polyclonal sheep anti-digoxigenin conjugated to alkaline phosphatase in 1% milk in PBS for 1 hour. After washing, the hybridised cells were visualised by enzyme-catalysed colour reaction using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium salt (Boehringer Mannheim).

**RNA extraction and northern blots**

Purified Schwann cells were cultured in 75 cm² tissue culture flask coated with polylysine and laminin as described above. BALB/c 3T3 cells were cultured in uncoated flasks. Schwann cells were treated with forskolin and growth factors as described. Alternatively, the purified Schwann cells were treated with 4 μM forskolin and semi-pure glial growth factor in DMEM containing 10% calf serum for 6 days to increase the cell numbers, then placed in DMEM with 10% calf serum for 4 days prior to the start of the experiment. Total RNA was extracted from desheathed sciatic nerves of postnatal day 6 rats, Schwann cell cultures or BALB/c 3T3 cell cultures by the method of Chomczynski and Sacchi (1987). The cDNA used for northern blots was excised from plasmid pSN63c using EcoRI (Lemke and Axel, 1985). The cDNA was labelled using a Boehringer Mannheim random primed DNA-labelling kit, following the manufacturers instructions. Unincorporated deoxyribo-nucleoside triphosphates were removed by chromatography through a Bio-Spin column (Biorad). The RNA was then run on an agarose/formaldehyde gel and transferred to a nylon membrane (Genescreen, DuPont UK). This was hybridised with 32P-CTP-labelled cDNA (Amersham International plc) (specific activity ranged from 1-5x10⁶ dpm pmol⁻¹ and the probe was used at 1-4x10⁵ dpm ml⁻¹) at 60°C for 16 hours with shaking. The nylon membrane was rinsed, washed for 1 hour in 2x SSC with 1% SDS at 60°C, followed by 1 hour in 0.1x SSC at room temperature, before exposing to X-ray film (Kodak XAR-2).

**Immunocytochemistry for P0 and BrdU**

For P0 labelling, cells were fixed in 2 M HCl at room temperature for 20 minutes. After washing in PBS, they were incubated for 1-2 hours in antibody diluting solution (PBS containing 10% FCS, 0.1 M lysine and 0.02% sodium azide). Cells were then incubated in P0 antibody in the same solution overnight. The cells were subsequently washed and incubated in donkey anti-rabbit Ig conjugated to biotin for 1 hour, followed by streptavidin-fluorescein for 20 minutes.

DNA synthesis was measured in double labelling experiments using P0 antibodies combined with BrdU antibodies. BrdU was added to the cultures at a concentration of 2.5x10⁻⁵ M for the final hour of culture (Gratzner, 1982). In double labelling experiments with P0 antibodies, cells were washed, fixed with 2 M HCl for 20 minutes to denature DNA and washed for 10 minutes in 0.1 M sodium borate (pH 8.5). Coverslips were incubated sequentially with (i) BrdU antibodies in PBS containing 0.1% Triton X-100 for 2 hours, (ii) goat anti-mouse Ig (Amersham, UK) at 37°C for 16 hours, (iii) donkey anti-rabbit Ig conjugated to biotin for 1 hour, (iv) streptavidin-fluorescein, as above. All coverslips were mounted on microscope slides in Citifluor anti-fade mounting medium (City University) and viewed on a Zeiss fluorescence microscope with epifluorescence or phase-contrast optics. S100 immunohelabelling was carried out as described previously (Morgan et al., 1991).

**Immunoblotting**

Purified Schwann cells were cultured in 25 cm² tissue culture flasks coated with polylysine and laminin. BALB/c 3T3 cells were cultured in uncoated flasks. Schwann cells were seeded at 5x10⁵ cells per flask and treated with forskolin, forskolin with FGF2 or TGFβ1, TGFβ2 or TGFβ3, or growth factors alone for 3 days, in defined medium as described above. Control cultures were maintained without addition of forskolin or growth factors. Proteins were extracted from the cells or sciatic nerve, subjected to SDS-PAGE electrophoresis and Western blotting to the second, made in this laboratory essentially as described by Brockes et al. (1980), was used diluted 1:500 for immunocytochemistry and 1:1,000 for immunoblotting. Streptavidin-peroxidase conjugated (Amersham International plc) (specific activity ranged from 1-5x10⁶ dpm pmol⁻¹ and the probe was used at 1-4x10⁵ dpm ml⁻¹) at 60°C for 16 hours with shaking. The nylon membrane was rinsed, washed for 1 hour in 2x SSC with 1% SDS at 60°C, followed by 1 hour in 0.1x SSC at room temperature, before exposing to X-ray film (Kodak XAR-2).

**RESULTS**

In short-term Schwann cell cultures in defined medium P0 mRNA is elevated by agents that mimic or elevate intracellular cAMP

To determine the time course of P0 mRNA induction in
response to cAMP elevation in defined medium, Schwann cells, cultured for 5 days, were treated with 4 μM forskolin for 12, 24 and 36 hours prior to fixation and probing with a digoxigenin-labelled riboprobe using in situ hybridisation (Fig. 1). Counts of P₀ mRNA-positive cells in these cultures revealed a clear elevation of P₀ mRNA at 12 hours with the number of labelled cells levelling off between 24 and 36 hours at 50-60% (the numbers of positive cells at these two time points were not significantly different). When cells were treated with digoxigenin-labelled mRNA transcribed in the sense orientation no labelling was seen (data not shown). In comparison, when P₀ protein was detected using antibody and immunofluorescence in cultures treated in identical fashion no P₀-positive cells were detected at 24 hours, 27±17.1% were positive at 48 hours and maximal levels (51±5.7%) were seen at 3 days after onset of treatment (see also Morgan et al., 1991).

These results were confirmed by exposing similar cultures to a mixture of 8 bromo and dibutyryl cAMP or to cholera toxin, to mimic elevation of intracellular cAMP levels, for 24, 36, 48 and 72 hours prior to fixing and probing with a 35S-UTP-labelled P₀ oligonucleotide probe (not shown).

**Serum suppresses induction of P₀ mRNA**

In view of our previous finding that serum factors sharply reduce the amount of P₀ protein that is induced by cAMP elevation, we used northern blots to examine the amount of P₀ mRNA induced by exposure to 4 μM forskolin for 48 hours in the presence and absence of 10% calf serum (Fig. 2).

Greatly increased levels of P₀ mRNA were seen in blots of cultured cells in defined medium treated with 4 μM forskolin without serum for 48 hours. Very low levels of P₀ mRNA were detectable in cells cultured in defined medium alone, indicating that even in Schwann cells cultured in the absence of agents that elevate intracellular cAMP levels, the level of P₀ mRNA does not fall to zero, confirming observations made using in situ hybridisation.

Inclusion of serum consistently resulted in obvious reduction in the amount of P₀ mRNA, although the magnitude of this effect was variable, ranging from essentially a complete block to a substantial reduction. The range of results probably reflects dependence on unknown factors in serum that are not tightly controlled in these experiments.

These observations partially reconcile our previous finding that cAMP-induced P₀ protein expression is very much reduced in the presence of serum with the observations of others that cAMP elevation in serum clearly increases P₀ mRNA levels in Schwann cells (Lemke and Chao, 1988; Kambholz et al., 1992; Mews and Meyer, 1993).

**FGF2 and TGFβs suppress induction of P₀ mRNA**

The negative regulation of P₀ gene expression seen with serum was explored further by testing whether purified recombinant factors present in peripheral nerves in vivo had similar effects. Using defined medium without serum, cultured Schwann cells were exposed to 4 μM forskolin for 24 hours in the presence or absence of 10 ng ml⁻¹ FGF2, 1 ng ml⁻¹ TGFβ1, 1 ng ml⁻¹ TGFβ3 or 1 ng ml⁻¹ TGFβ1 (data not shown for TGFβ3). P₀ mRNA abundance was monitored by northern blotting or in situ hybridisation (Figs 3, 4). In every case, these growth factors were able to reduce the amount of P₀ mRNA induced by forskolin exposure, with the most effective being TGFβ3 and TGFβ1 (Fig. 4). This suppression was dose dependent, with 10 ng ml⁻¹ TGFβ3 reducing P₀ mRNA levels to 13% of control values (data not shown).

**Northern blot analysis**

Northern blot analysis. Total RNA extracted from postnatal day 6 sciatic nerve and from cultured cells. Lanes are labelled according to the source of the RNA. SN, sciatic nerve; 3T3, fibroblast cell line cultured in 10% calf serum; Def med, Schwann cells in defined medium; Forsk, Schwann cells treated with 4 μM forskolin in defined medium for 48 hours; CS, Schwann cells in 10% calf serum; CS + Forsk, Schwann cells treated with 4 μM forskolin for 48 hours in 10% calf serum. RNA loadings are visualised in the lower panel. 2 μg of sciatic nerve RNA and 10 μg of RNA from cultured cells loaded per track.
Fig. 4. FGF2 and TGFβs suppress induction of P0 mRNA: in situ hybridisation. P0 mRNA in Schwann cells was visualised using a digoxigenin-labelled riboprobe after 24 hours treatment with 4 µM forskolin alone or forskolin and growth factors as indicated. FGF2 was used at 10 ng ml−1 and TGFβ2 and TGFβ3 at 1 ng ml−1. The cells are shown with bright-field (left-hand panels) and phase-contrast optics (right-hand panels). Bar 20 µM.
factors strongly suppressed $P_o$ gene expression in the presence of forskolin.

The dose-response relationship of this effect was investigated using FGF2 or TGFβ2 plus forskolin in defined medium (Fig. 5). For FGF2 substantial inhibition was seen at 0.1 ng ml$^{-1}$ with maximal suppression at 10 ng ml$^{-1}$ ($P<0.01$, Student’s t test). For TGFβ2, maximal suppression was already seen at 0.1 ng ml$^{-1}$ ($P<0.01$, Student’s t test). In control experiments, 10 ng ml$^{-1}$ FGF2 and 1 ng ml$^{-1}$ TGFβ2 were applied to Schwann cells without forskolin for 24 hours in defined medium. No obvious effects on basal $P_o$ mRNA levels were noted.

**FGF2 and TGFβ1, TGFβ2 and TGFβ3 suppress $P_o$ protein expression**

To test whether the suppressive effect of these factors on $P_o$ mRNA expression was evident at the level of $P_o$ protein expression, Schwann cell cultures were exposed to 4 μM forskolin for 72 hours in the presence or absence of 10 ng ml$^{-1}$ FGF2 or 1 ng ml$^{-1}$ TGFβ1, TGFβ2 or TGFβ3 using defined medium. Assessment of $P_o$ protein levels using immunoblotting showed a strong induction of $P_o$ protein by forskolin alone, and a substantial reduction in protein levels in the presence of each of the growth factors (Fig. 6). Confirmation of this effect was obtained in other experiments in which $P_o$ protein expression was monitored by immunohistochemistry (see below).

**FGF2 inhibition of $P_o$ expression does not depend on stimulation of DNA synthesis**

We found previously that reduction of $P_o$ protein expression was strongly correlated with the passage of cells through the cell cycle and DNA synthesis, suggesting that the suppression of the $P_o$ gene might be secondary to, or downstream from, the mitotic response. We now investigated this issue for the FGF2-mediated inhibition of $P_o$ expression.

First, we found that increasing the concentration of FGF2 in the presence of a constant amount of forskolin, is indeed accompanied by an increase in the number of Schwann cells in the cell cycle as judged by the percentage of cells synthesizing DNA. This result is consistent with previous work on the mitogenic effect of FGF2 plus forskolin in defined medium (Stewart et al., 1991) (Fig. 7). Similar results were obtained with FGF1 in the presence of heparin (data not shown).
We now asked whether the FGF2 inhibition of \( P_b \) expression depended on the mitogenic effect, by testing whether the number of cells expressing \( P_b \) protein in the presence of 4 \( \mu M \) forskolin was reduced by FGF2 only under mitogenic conditions, i.e. conditions that cause serum starved quiescent Schwann cells to enter the cell cycle and synthesize DNA. In these experiments, we took advantage of the finding that the combination of FGF2 and forskolin is not mitogenic for Schwann cells if the insulin concentration is dropped from the routine 5 \( \mu g \) ml\(^{-1}\) to 5 ng ml\(^{-1}\), a concentration that will activate insulin, but not IGF receptors (Neely et al., 1991; Stewart et al., 1991; Schumacher et al., 1993; H. J. S Stewart, K. R. Jessen and R. Mirsky, unpublished observations). We found, firstly, that reducing the insulin concentration to 5 ng ml\(^{-1}\) did not reduce the number of cells induced by forskolin to express \( P_b \) and, secondly, that 10 ng ml\(^{-1}\) FGF2 caused clear and statistically significant suppression of \( P_b \) expression even when applied in 5 ng ml\(^{-1}\) insulin (\( P < 0.01 \), Student’s \( t \) test), conditions that did not stimulate DNA synthesis. Thirdly, we found that a further inhibition was obtained when FGF2 was applied in 5 \( \mu g \) ml\(^{-1}\) insulin, in which case the cells were also stimulated to enter the cell cycle and synthesize DNA (\( P < 0.01 \) Student’s \( t \) test, comparing high and low insulin) (Fig. 8).

Lastly, we used 300 \( \mu M \) thymidine, which holds cells at the G1/S transition (Bjursell and Reichard, 1973; Tobey et al., 1988), to analyse further the relationship between FGF2 inhibition of \( P_b \) expression and cell cycle events. Having found that 300 \( \mu M \) thymidine alone does not interfere with the ability of 4 \( \mu M \) forskolin to induce \( P_b \) protein (Fig. 9), we examined the FGF2 block of \( P_b \) protein expression in the presence and absence of thymidine (Fig. 9). We found that although inclusion of thymidine significantly reduced the FGF2-mediated block of \( P_b \) protein expression, suppression was still evident.

Thus, FGF2 suppresses \( P_b \) expression even in serum-starved, quiescent Schwann cells. The same concentration of FGF2 reduces \( P_b \) expression even further if it is applied under conditions that simultaneously allow Schwann cells not only to enter but also to traverse the cell cycle.

**TGFβs block \( P_b \) expression without significant stimulation of DNA synthesis**

Strong additional evidence for a negative regulation of the \( P_b \) gene that operates in quiescent Schwann cells, and is therefore unlikely to be secondary to a mitogenic response, was obtained in experiments with TGFβs.

In experiments described above, we showed that TGFβs...
suppressed Po mRNA and protein expression in the presence of 4 µM forskolin. Since, in contrast to FGF2 plus forskolin, the combination of TGFβs and forskolin is not mitogenic for Schwann cells according to previous reports (Stewart et al., 1991; Schubert, 1992) those observations already pointed to the independence of TGFβ-mediated Po inhibition from cell division. To demonstrate this unambiguously, we studied Po gene expression at the mRNA and protein level in the presence of 4 µM forskolin in the presence or absence of TGFβs, monitoring DNA synthesis at the same time (Fig. 10). The results showed that 0.1 µg ml⁻¹ TGFβ2 completely blocked Po protein expression in the virtual absence of DNA synthesis (Fig. 10A). To confirm that DNA synthesis was extremely low over the whole period of the experiment, DNA synthesis in the presence of 4 µM forskolin and 0.1 ng ml⁻¹ TGFβ2 was measured over the whole 72 hours. It averaged 0.48±0.15%, n=9, over the first 24 hours after addition of drugs, 1.58±0.41%, n=6 over the second 24 hour period, and 6.12±1.36%, n=6 over the third 24 hour period. Removal of TGFβ2 resulted in upregulation of Po protein to levels seen in the presence of forskolin alone within 72 hours. Using ten-fold higher concentrations of TGFβ1, TGFβ2 and TGFβ3, an essentially complete block of Po mRNA or protein expression accompanied by minimal DNA synthesis was seen (Fig. 10B).

**DISCUSSION**

We have demonstrated that elevation of cAMP levels in short-term Schwann cells cultured in serum-free defined medium induces elevation of Po mRNA within 12 hours with maximal numbers of cells induced by 24 hours. Elevation of Po mRNA and protein by forskolin can be strongly suppressed by either FGFs or TGFβs.

For both growth factor families, this inhibition of Po gene expression is strong, dose-dependent and can take place in serum-starved cells that are mitotically quiescent. In the case of FGF2, however, even more complete suppression is obtained when the cells are simultaneously allowed to enter and move through the cell cycle.

Since both FGFs and TGFβs are present in developing nerves (Kalcheim and Neufeld, 1990; Flanders et al., 1991; Unsicker et al., 1991; Scherer et al., 1993), it is feasible to suggest that myelination may be subject to negative regulation by these growth factors during normal development. Po gene expression and myelination by Schwann cells is generally envisaged as being under the positive regulation of axon-associated signals, the molecular nature of which remains unclear. Many lines of evidence indicate that such signals instruct Schwann cells to switch on the genes for myelin proteins, to switch off genes for the protein markers of non-myelinating Schwann cells, and to carry out the membrane synthesis and wrapping events necessary for the formation of a myelin sheath. The present results raise the possibility that the onset, rate and determination of these events is a function not only of the presence and effectivity of these instructive signals, but reflects a balance between positive and negative regulation.

**Elevation of Po mRNA in defined medium**

The elevation of Po mRNA in serum-free conditions in which the cells do not divide parallels the large induction of Po protein that we have previously observed under similar conditions but substantially precedes it in time (Morgan et al., 1991). It is also greater than when Po mRNA is induced in the presence of serum, suggesting that serum has suppressive effects not only on Po protein induction, as we showed previously (Morgan et al., 1991) but also at the mRNA level. These observations and those of other laboratories that forskolin elevates Po mRNA in serum-containing media (Lemke and Chao, 1988: Kamholz et al., 1992; Mews and Meyer, 1993), are therefore compatible, particularly when it is taken into account that other studies have used higher forskolin concentrations that are likely to overcome the serum inhibition more effectively.

A recent paper on induction of myelination in
DRG/Schwann cell co-cultures also describes serum-mediated inhibition of P₀ mRNA induction, caused in this case by axon-associated signals. This effect can be overridden by addition of ascorbate to the serum-containing medium. Under these conditions, basal lamina formation and P₀ mRNA induction and myelin formation occurs in Schwann cells surrounded by large axons, whereas P₀ mRNA induction in non-myelin-forming Schwann cells remains suppressed (Fernandez-Vallée et al., 1993).

Both FGF2 and TGFβs suppress the induction of P₀ mRNA
Both FGF2 and TGFβs suppress the elevation of P₀ mRNA induced by elevation of cAMP to a highly significant extent. In the case of TGFβ2, a concentration of TGFβ2 (0.1 ng ml⁻¹) that induces minimal DNA synthesis causes an essentially complete block of P₀ mRNA and protein induction in the presence of 4 μM forskolin. The results reported here show that even at higher doses of TGFβ there is little DNA synthesis in response to forskolin at 72 hours, confirming our previous results showing that, in defined medium, TGFβ and forskolin did not cause DNA synthesis in Schwann cells (Stewart et al., 1991). Therefore DNA synthesis and progression through the cell cycle is not required for the suppression of P₀ mRNA induction by TGFβs. In other systems, it has been reported that TGFβs lengthen the G₁ phase of the cell cycle rather than cause progression through to S phase (Moses et al., 1990; Zentella and Massague, 1992). These experiments demonstrate that, even in quiescent cells, it is possible to block the induction of P₀ caused by elevation of intracellular cAMP levels. Our results with TGFβ2 and TGFβ3 parallel and extend those of Mews and Meyer (1993) who reported that administration of TGFβ1 in the presence of serum and forskolin suppressed P₀ mRNA induction in cultured neonatal Schwann cells at 24 hours, although at 3 days no suppression was observed, perhaps because, as they suggest, the TGFβ was not replaced daily, whereas in our experiments it was replaced daily. P₀ protein expression and DNA synthesis were not measured in their experiments. They suggested the effects of TGFβ on Schwann cells treated with forskolin were compatible with partial induction of a non-myelin-forming phenotype, since both P₀ mRNA and p75 NGFR mRNA were suppressed. The parallels are far from complete, however, since the lipids galactocerebroside and 04, both found on non-myelin-forming Schwann cells and induced in cultured Schwann cells in response to forskolin, are not induced in cultured Schwann cells treated with TGFβs and forskolin (H. J. S. Stewart, G. Rougon, R. Mirsky and K. R. Jessen, unpublished data).

A dissociation between conditions that suppress P₀ mRNA levels on the one hand and stimulate DNA synthesis on the other is also seen when FGF2 is applied in the presence of low insulin concentrations. Since even more complete suppression is seen when insulin levels are elevated and DNA synthesis consequently stimulated, it is possible that two mechanisms for suppression exist, one of which requires progression through the cell cycle and another which does not.

In the case of FGF2, where DNA synthesis levels in the presence of forskolin are substantial, the evidence that progression through the cell cycle is required for suppression is not conclusive. Experiments using thymidine indicate that mitosis prior to induction is not required since FGF2 can still suppress P₀ protein induction even in the presence of thymidine. Thymidine has, however, been reported to inhibit events at or just after the onset of the G₁/S transition (Bjursell and Reichard, 1973; Tobey et al., 1988; Hohmann et al., 1993). It is therefore impossible from this experiment to be sure that entry into early S phase is not required for the suppressive effect of FGFs. When DNA synthesis is suppressed by lowering the insulin level so that it cannot activate IGF receptors, the suppressive effect of FGF2 is still observed, but it is partial. Therefore, in the case of FGF2, progression to the G₁/S boundary increases the efficiency of suppression.

Comparison with other systems
Our results raise obvious parallels with muscle differentiation. In muscle, both FGFs and TGFβs can prevent myoblast differentiation into myotubes (Olson et al., 1986; Spizz et al., 1986; Massagué, 1990; Olson, 1992; Zentella and Massagué, 1992). In muscle, it is important for cells to be at low density for down-regulation of muscle-specific genes in response to growth factors, including TGFβ and FGF (Hu and Olson, 1990), an effect that we also observed with the down-regulation of P₀ mRNA in Schwann cells in the case of FGF, where cells plated at 20,000 cells/well clearly showed lower levels of suppression than cells that were plated at 10,000 cells/well (data not shown).

In the muscle system, FGF induces phosphorylation of the myogenic helix-loop-helix (HLH) proteins which control muscle-specific transcription (Edmonson and Olson, 1993). The phosphorylation prevents binding of the HLH transcription factors, including myogenin and MyoD, to DNA, which in turn prevents activation of muscle differentiation-specific genes (Li et al., 1992). It has also been demonstrated that in muscle the commitment to differentiation involves interaction between the myogenic HLH proteins and the retinoblastoma protein which is important in regulating entry into the cell cycle (Gu et al., 1993). MyoD also binds to, and is repressed by c-Jun (Bengal et al., 1992). It is tempting to speculate that either FGF- or TGFβ-mediated suppression of P₀ mRNA induction may involve related mechanisms in Schwann cells. C-Jun is expressed in cultured Schwann cells and is down-regulated by cAMP, but in transfection studies does not bind directly to the P₀ promoter. The Schwann cell transcription factor SCIP, however, both binds and represses the promoter so it might be involved in some unknown way (Monuki et al., 1989; 1990; 1993; He et al., 1990). In the case of TGFβ, another possible factor that could be involved is the cAMP response element binding protein (CREB) since TGFβ1 can phosphorylate CREB, apparently at a different site from that phosphorylated by protein kinase A in response to cAMP elevation (Kramer et al., 1991). TGFβ might thus act to prevent CREB phosphorylation by protein kinase A, which is required for binding to the CRE element and transcriptional activation of CRE-containing genes (Gonzalez et al., 1991). Since, however, neither P₀ or other myelin proteins are reported to contain CRE-containing sequences any interaction between CREB/CRE and myelin genes must be indirect (Lemke et al., 1988).
growth factors. Such factors are present in developing nerves raising the possibility that a complex series of negative regulatory events play a part in the overall control of Schwann cell myelination. The intracellular mechanisms by which this is achieved will be the subject of further studies.

We thank B. D. Trapp, M. Jones and D. Mason for antibodies, and M. Jaye for FGFI, and I. Griffiths, G. Lemke, L.D. Hudson and M. J.Lee for DNA constructs and oligonucleotides. Thanks also to Mrs D. Bartram for secretarial help. This work was supported by grants from Action Research for the Crippled Child and the Medical Research Council of Great Britain.

REFERENCES


(Accepted 17 March 1994)
1410 Morgan, K. R. Jessen and R. Mirsky