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Cell-specific cyclic AMP-mediated induction of the PDGF receptor

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Cyclic AMP (cAMP) cooperates with a wide variety of polypeptide growth factors to synergistically stimulate the proliferation of many vertebrate cell types. However, the cellular mechanisms underlying these cooperative interactions are for the most part unknown. We have identified one such mechanism by observing that (i) cultured rat Schwann cells proliferate in response to platelet-derived growth factor (PDGF) only if simultaneously cultured in the presence of agents that elevate intracellular cAMP and (ii) this unmasked PDGF response is accounted for by a dramatic cAMP-mediated induction of PDGF receptor mRNA and protein. cAMP-mediated induction of the PDGF receptor results in enhanced, ligand dependent receptor autophosphorylation, and in enhanced PDGF activation of c-*fos* gene expression. In addition, this induction is unique to those cells, such as Schwann cells, for which cAMP is itself mitogenic. These results indicate that the synergistic proliferative effect obtained from the combination of cAMP and polypeptide growth factors may in large result from the cAMP-mediated induction of growth factor receptors.

Key words: cAMP/cell proliferation/PDGF/PDGF receptor/Schwann cells

Introduction

Cyclic AMP (cAMP) is capable of exerting both positive and negative effects on mammalian cell division, depending upon the cell type assayed (for review, see Dumont *et al.*, 1989). For many fibroblast and established tumor cell lines, this second messenger is either without effect or is a negative regulator of proliferation (Pastan *et al.*, 1975). In contrast, skin and mammary epithelia (Green, 1978; Yang *et al.*, 1980), hepatocytes (Friedman *et al.*, 1981), thyrocytes (Roger *et al.*, 1983), Schwann cells (Raff *et al.*, 1978a) and melanocytes (Mayer, 1982), among many other cell types, respond to elevation of intracellular cAMP by dividing. For these cells, combined application of cAMP and a polypeptide mitogen, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) or insulin, typically promotes a synergistic (greater than additive) stimulation of cell proliferation (Raff *et al.*, 1978a; McGowan *et al.*, 1981; Roger and Dumont, 1984; Westermark *et al.*, 1986; Roger *et al.*, 1987). An explanation for this widely observed cooperativity has thus far remained elusive.

We have used purified cultured Schwann cells, the principal glial cells of the peripheral nervous system (PNS), to investigate the mechanisms underlying the synergistic interaction of cAMP and polypeptide growth factors. We have measured Schwann cell proliferation, gene expression and protein tyrosine kinase activity in response to the polypeptide growth factor PDGF, both in the presence and absence of forskolin, a specific and reversible activator of the cAMP-producing enzyme adenyl cyclase (Seamon *et al.*, 1981). Our results suggest a straightforward explanation for cAMP–PDGF cooperativity; cAMP is capable of strongly inducing the gene encoding the PDGF receptor. This induction results in enhanced early and late responses to PDGF, and is unique to those cells for which mitogenic cooperativity between cAMP and PDGF is observed.

Results and discussion

PDGF is a cAMP-dependent Schwann cell mitogen

Although PDGF is a potent mitogen for fibroblasts and other mesenchymal cells (Ross *et al.*, 1986), it has previously been shown to be almost entirely ineffective as a mitogen for cultured rat Schwann cells. A very weak proliferative response to this growth factor has been observed only with unusually high concentrations (>100 ng/ml) of highly purified PDGF (Lemke and Brockes, 1984). Schwann cells do rapidly divide in response to the distinct polypeptide mitogen glial growth factor (GGF) (Lemke and Brockes, 1984), whose amino acid sequence has yet to be either deduced or directly determined. Since GGF is biochemically similar to PDGF (Lemke and Brockes, 1984), and since PDGF appears to regulate the division of central nervous system (CNS) glia (Raff *et al.*, 1988; Noble *et al.*, 1988), we asked whether this growth factor might act synergistically with cAMP to stimulate Schwann cell proliferation in a manner similar to that previously observed for the combination of cAMP and GGF (Raff *et al.*, 1978b; Porter *et al.*, 1986).

To test this hypothesis, we used a 48 h [^{125}I]iododeoxyuridine proliferation assay (Lemke and Brockes, 1984) to monitor DNA synthesis in cultured rat Schwann cells in response to increasing concentrations of either PDGF or the adenyl cyclase activator forskolin alone, or to increasing concentrations of PDGF added together with a fixed concentration of forskolin. In this and all subsequent experiments, we have used the BB homodimeric isoform of PDGF (Heldin *et al.*, 1985). The results of this proliferation analysis, illustrated in Figure 1, confirm that PDGF is a very poor mitogen when assayed alone. However, they further demonstrate that this growth factor is capable of strongly stimulating Schwann cell DNA synthesis, in a dose-dependent fashion, if assayed in the presence of forskolin. This cAMP-dependent PDGF stimulation is

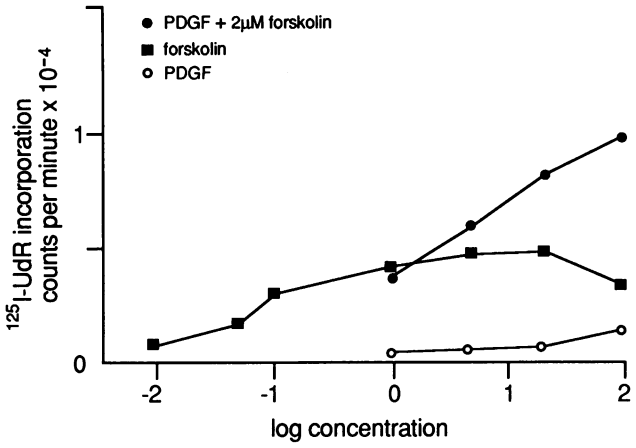

Fig. 1. Synergistic proliferative action of PDGF and cAMP. The proliferative effects of purified PDGF (BB homodimer) and forskolin alone and PDGF and forskolin together were measured in a 48 h Schwann cell radionucleotide incorporation assay (see Materials and methods). In this assay [^{125}I]UdR incorporation is a direct measure of DNA synthesis and cell division (Raff *et al.*, 1978b). Data points represent the average of triplicate determinations. PDGF concentrations: 1, 5, 20 and 100 ng/ml; forskolin concentrations: 10, 50, 100 and 500 nM, 1, 5, 20 and 100 μM. The same PDGF concentration range was assayed in the presence of 2 μM forskolin.

similar in dose–response to that previously described for the synergistic combination of GGF and forskolin (Porter *et al.*, 1986) or GGF and cholera toxin (Raff *et al.*, 1978b), and leads to a rapid expansion of cultured Schwann cell populations (data not shown).

cAMP elevation results in cell-specific induction of PDGF receptor mRNA

cAMP-induced Schwann cell responsiveness to PDGF is accompanied by a corresponding cAMP induction of mRNA encoding the PDGF receptor. As illustrated in the Northern blot of Figure 2A, 48 h treatment with forskolin, both in the presence and absence of GGF, results in a striking induction of mRNA encoding the PDGF receptor in Schwann cells. A similarly strong induction of PDGF receptor mRNA is evident following 48 h treatment with the cAMP analog dibutyryl cAMP (data not shown). Importantly, this induction does not occur in NIH3T3 fibroblasts (Figure 2B) or Rat-2 fibroblast (data not shown). PDGF receptor mRNA levels are constitutively high in these cells, and are slightly reduced upon elevation of intracellular cAMP (forskolin lane, Figure 2B). Unlike Schwann cells, these fibroblast lines do not exhibit a mitogenic response to cAMP elevation (Pastan *et al.*, 1975), but conversely, do actively divide in response to PDGF alone (Ross *et al.*, 1986). Consistent with both of these response properties is our observation that the PDGF receptor mRNA level present in untreated NIH3T3 cells (no addition lane, Figure 2B) is roughly equivalent to the up-regulated level of PDGF receptor mRNA expressed by forskolin-treated Schwann cells.

cAMP induction of many genes, including immediate-early response genes such as c-*fos* (Curran *et al.*, 1984), is characteristically rapid and transient (Greenberg *et al.*, 1985). cAMP induction of PDGF receptor mRNA exhibits neither of these properties. Instead, up-regulated levels of this mRNA are not observed until nearly 24 h after elevation of intracellular cAMP (Figure 3A), and once induced, these levels remain high so long as cAMP levels remain elevated.

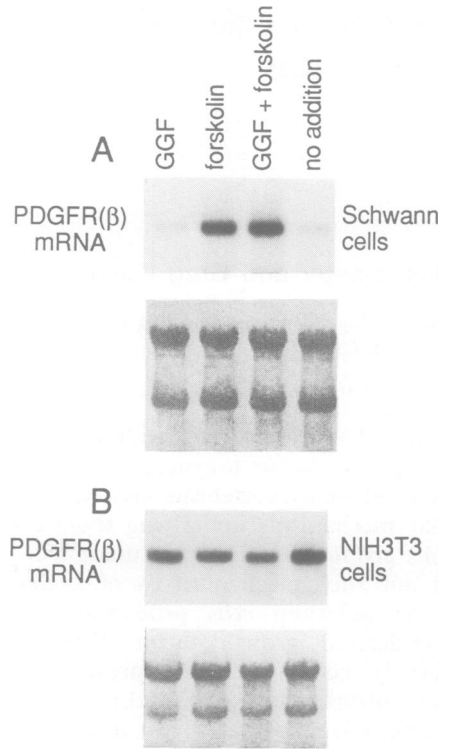

Fig. 2. The PDGF receptor gene is cAMP-inducible in cultured Schwann cells but not in fibroblasts. β-type PDGF receptor mRNA was measured in Schwann cells (**A**) and in NIH3T3 cells (**B**) grown for 48 h in culture medium alone (no addition), or in the presence of 20 μg/ml CM–GGF, 20 μM forskolin or GGF and forskolin together. **Upper panels** in A and B: Northern blots hybridized with a β-type PDGF receptor cDNA probe; **lower panels**: methylene blue staining following transfer. In this and subsequent blots, the relative amount of total RNA in each lane can be estimated from the methylene blue staining of 18S and 28S RNAs, performed prior to hybridization (see Materials and methods).

This delayed but persistent expression of PDGF receptor mRNA demonstrates that the PDGF receptor gene is not a typical immediate-early response gene, and suggests that cAMP induction occurs indirectly, at the end of an extended cascade of gene expression. If forskolin is removed from Schwann cells at either 1 or 3 h after its addition, no up-regulation of PDGF receptor mRNA levels is observed (Figure 3B). This result indicates that immediate-early response genes rapidly and transiently induced by cAMP are not by themselves sufficient to promote subsequent induction of PDGF receptor mRNA.

Each of the Northern blots illustrated in Figures 2 and 3 were hybridized with a cDNA probe that specifically recognizes mRNA encoding the β-type PDGF receptor (Yarden *et al.*, 1986). This receptor (also referred to as the B type) binds the BB PDGF isoform with high affinity, the AB isoform with reduced affinity and the AA isoform with very low affinity (Hart *et al.*, 1988; Heldin *et al.*, 1988). Recently, a second, α-type PDGF receptor, to which all three PDGF isoforms bind with high affinity, has been characterized and cloned (Claesson-Welsh *et al.*, 1989; Matsui *et al.*, 1989). Although mRNA encoding this α-type receptor is prominently expressed by both cultured fibroblasts and astrocytes, it is expressed at only extremely low levels by cultured Schwann cells (Figure 4). Peripheral glia are thus to be distinguished from their CNS counterparts which, by binding criteria (Hart *et al.*, 1989) and RNA

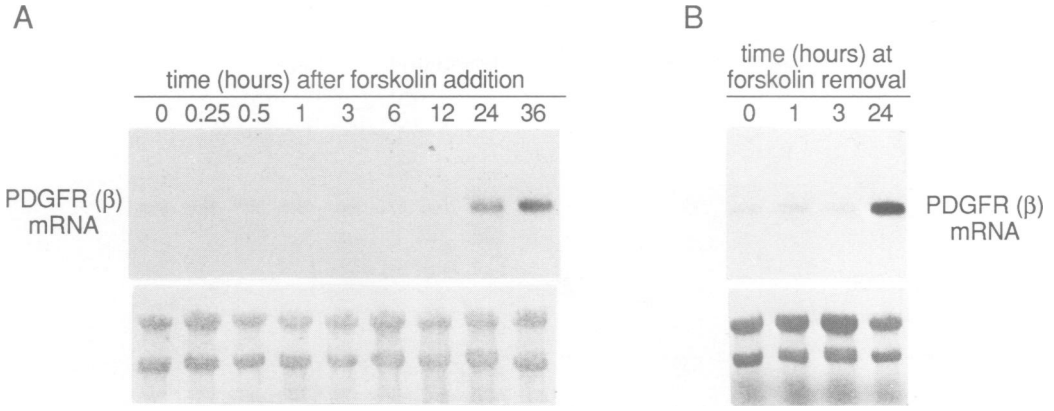

Fig. 3. cAMP up-regulation of PDGF receptor gene expression occurs with delayed kinetics, and requires continuous cAMP elevation. (**A**) Parallel plates of quiescent Schwann cells were exposed to 20 μM forskolin at time zero, and subsequently harvested for RNA isolation at the indicated times (in hours) following addition of the drug. (**B**) Parallel plates of quiescent Schwann cells were exposed to 20 μM forskolin at time zero. Cells were subsequently washed free of the drug at the indicated times (in hours), and all plates were then harvested for RNA isolation 24 h after the start of the experiment. **Upper panels** in (A) and (B): Northern blots hybridized with a β-type PDGF receptor cDNA probe. **Lower panels**: methylene blue staining following transfer.

hybridization (Figure 4), express high levels of both the α- and β-type PDGF receptors.

Schwann cells expressing cAMP-elevated levels of PDGF receptor exhibit enhanced PDGF-dependent signal transduction

As illustrated in the Western blot of Figure 5A, cAMP-induced β-type PDGF receptor mRNA is translated into a correspondingly elevated level of protein, as detected by an anti-peptide antiserum that specifically recognizes the PDGF receptor (Keating and Williams, 1987). Schwann cells cultured for 48 h in 20 μM forskolin (+F lane) express >10 times the amount of the 180 kd PDGF receptor protein (arrowed) than do Schwann cells cultured in medium not supplemented with forskolin (−F lane).

By two criteria, this induced receptor protein is expressed on the surface of Schwann cells and is functional. First, it undergoes rapid tyrosine autophosphorylation in response to exogenous PDGF (compare NT^f and $PDGF^f$ lanes, Figure 5B), as detected by anti-phosphotyrosine antibodies (Kamps and Sefton, 1988). Characteristically, this autophosphorylation occurs within 5 min of PDGF addition and is specifically reduced by 5 h prior incubation with 50 ng/ml PDGF, a pre-treatment that results in receptor down-regulation (compare $PDGF^f$ and $PDGF^f$/DR lanes) (Heldin *et al.*, 1982; Rosenfeld *et al.*, 1984). Most importantly, PDGF-induced receptor autophosphorylation is strikingly higher in forskolin-treated as opposed to untreated Schwann cells (compare $PDGF^f$ and PDGF lanes). A second criterion for surface expression of functional protein is that cAMP induction of PDGF receptors is reflected in enhanced, PDGF-dependent expression of the proto-oncogene c-*fos*, an immediate-early transcription factor that has been widely implicated as an early mediator of mitogenic and differentiation stimuli (Curran and Morgan, 1987). As illustrated in the Northern blot of Figure 6, rapid and transient PDGF-dependent induction of c-*fos* mRNA is more pronounced in Schwann cells expressing high levels of PDGF receptor as a result of 48 h forskolin pre-treatment (FPT lanes) than in non-forskolin-treated cells expressing low levels of the receptor (NPT lanes). These results indicate that the number of PDGF receptors expressed on the surface

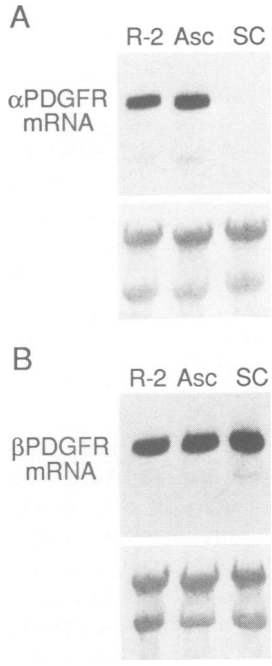

Fig. 4. PDGF stimulation of Schwann cell proliferation is mediated through the β-type and not the α-type PDGF receptor. Total cellular RNA from Rat-2 fibroblasts (R-2 lanes), rat cortical astrocytes (Asc lanes) or forskolin-treated rat Schwann cells (SC lanes) was analyzed by Northern blot for expression of α-type PDGF receptor mRNA (**A**) and β-type PDGF receptor mRNA (**B**). Very long (2 week) exposure of the autoradiogram in (A) reveals a very faint α-type PDGF receptor band in the SC lane. This level of expression (<1% of the β-type mRNA level) is too low to be detected in the 19 h exposure presented. **Lower panels** in (A) and (B): methylene blue staining following transfer.

of non-forskolin-treated Schwann cells, although low, is sufficient to trigger an appreciable PDGF-induced c-*fos* response. However, this low number of receptors is insufficient to trigger a maximal response.

A simple mechanism for mitogenic cooperativity

Taken together, these observations suggest a general mechanism whereby cAMP may effectively regulate the cellular response to polypeptide growth factors through

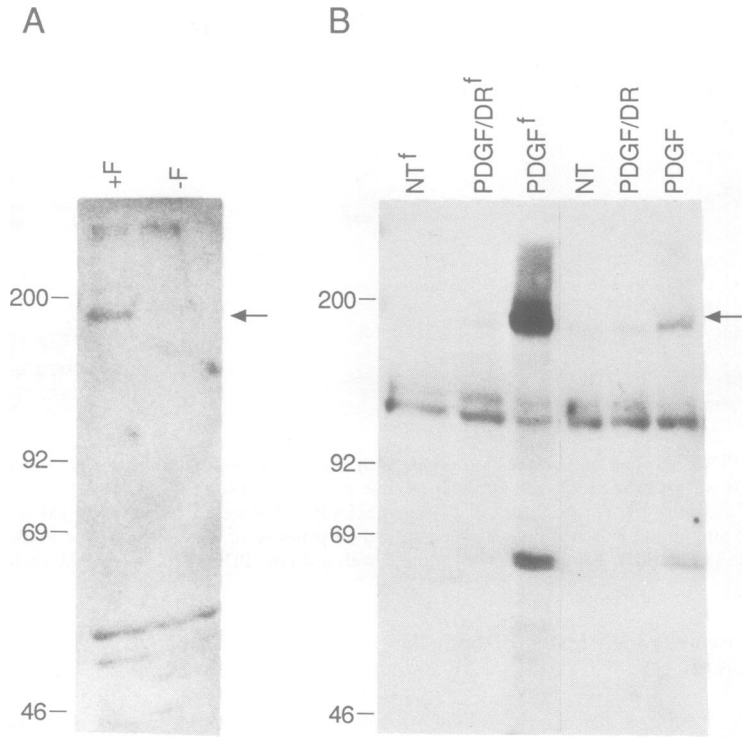

Fig. 5. cAMP elevation results in elevated surface expression of functional PDGF receptor protein. (**A**) Rat Schwann cells were cultured in either DMEM + 1% FBS alone (−F) or in this medium supplemented with 20 μM forskolin (+F) for 48 h. Cells were then harvested and analyzed by Western blot analysis as described in Materials and methods. PDGF receptor protein was identified with a specific anti-receptor antibody (Keating and Willams, 1987) and subsequent incubation with [^{125}I]protein A, as described in Materials and methods. The 180 kd PDGF receptor band is arrowed. (**B**) Parallel plates of rat Schwann cells were cultured in either DMEM + 1% FCS alone (three plates) or in this medium supplemented with 20 μM forskolin (three plates) for 48 h. One plate in each set was left untreated (NT and NT^f lanes); one plate in each set was treated with 50 ng/ml PDGF for 5 h prior to exposure to 50 ng/ml fresh PDGF for 5 min (PDGF/DR and PDGF/DR^f lanes); the final plate in each set was exposed to 50 ng/ml PDGF for 5 min without prior exposure to the growth factor (PDGF and PDGF^f lanes). ^{f} indicates those cells exposed to 20 μM forskolin for 48 h prior to the start of the experiment. Following the 5 min exposures to PDGF, all plates were harvested and processed as described in Materials and methods. Cell lysates were electrophoresed and blotted as for (A). Western blots were probed with an anti-phosphotyrosine antibody (Kamps and Sefton, 1988) and secondarily with [^{125}I]protein A. The autophosphorylated PDGF receptor protein is arrowed.

induction of growth factor receptors. As noted above, proliferative synergy between cAMP and growth factors has been observed for a variety of cells of both neural and non-neural lineage. This synergy distinguishes these cells from an equally large set of cultured mesenchymal derivatives for which cAMP is not mitogenic. We suggest that as a general rule, mitogenic cooperativity between cAMP and polypeptide growth factors such as GGF, EGF, PDGF, FGF and insulin is likely to result in part from cAMP induction of the corresponding growth factor receptor. Induction of these receptors may further account for the widely observed mitogenicity of cAMP alone, since *in vitro* proliferation assays are typically conducted in the presence of serum, which contains low concentrations of PDGF and other polypeptide growth factors. In this regard, Raff *et al.* (1978a) have previously noted that elevation of intracellular cAMP does not trigger Schwann cell division if these cells are cultured in medium containing ≤1% FBS. It may be of significance that each of the growth factors that exhibit mitogenic cooperativity with cAMP binds to a receptor that is known to possess intrinsic protein tyrosine kinase activity (Williams, 1989). The exception is GGF, whose receptor is as yet uncharacterized.

During development, the regulated response to polypeptide growth factors appears to play a central role in early events such as mesodermal induction (Kimelman and Kirschner, 1987), as well as in the timing and specification of cell

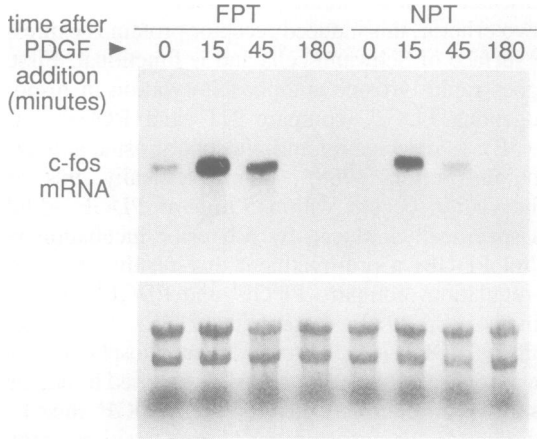

Fig. 6. cAMP elevation results in enhanced PDGF-dependent c-*fos* induction. Parallel plates of Schwann cells were cultured for 48 h in either DMEM + 2% FBS alone (four plates, NPT lanes) or in this medium supplemented with 20 μM forskolin (four plates, FPT lanes). All plates were then exposed to 50 ng/ml PDGF at time zero and were subsequently harvested for RNA isolation at the indicated times (in min) following PDGF addition. **Upper panels**: Northern blots hybridized with a *fos* cDNA probe; **Lower panels**: methylene blue staining following transfer.

lineage in the nervous system and elsewhere (Raff *et al.*, 1988). Synergistic cooperativity between cAMP and growth factor-stimulated protein kinase C has also been recently

shown to regulate neural induction in *Xenopus* embryos (Otte *et al.*, 1989). Given the results presented above, a direct role for cAMP regulation of each of these developmental events should be considered.

Materials and methods

Materials

The CM (carboxymethyl)−cellulose fraction of glial growth factor (CM−GGF) was prepared from lyophilized bovine anterior pituitary lobes as described by Lemke and Brockes (1984). Although only partially purified, all of the rat Schwann cell mitogenic activity present in this fraction is due to the 31 kd GGF protein (Brockes *et al.*, 1980; Lemke and Brockes, 1984). PDGF BB homodimer (recombinant c-*sis*) and forskolin were purchased from AMGen and Calbiochem respectively. Cell culture media and sera were purchased from Gibco. [^{125}I]Iododeoxyuridine (2200 Ci/mmol) and [^{125}I]protein A (100 Ci/μg) were purchased from New England Nuclear. The following cDNA probes were used: *fos*, the 1089 bp *Ava*I fragment of the v-*fos* clone described by Van Beveren *et al.* (1983); α-type PDGF receptor, a 1.3 kb *Eco*RI−*Eco*RV fragment of the rat α-type cDNA (K.-H.Lee and R.Reed, personal communication); β-type PDGF receptor, the 1161 bp *Hin*cII fragment of the mouse β-type receptor cDNA described by Yarden *et al.* (1986). The following antibodies were used: the rabbit anti-PDGF receptor antiserum 88 prepared and characterized by Keating and Williams (1987), and the rabbit anti-phosphotyrosine antiserum prepared and characterized by Kamps and Sefton (1988).

Schwann cell culture

Rat Schwann cells were prepared by dissociation from neonatal (2−3 day) rat sciatic nerve, and purified by immunoselection using anti-Thy1.1 and complement, as described by Brockes *et al.* (1979). Purified Schwann cell populations were expanded by growth in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 μM forskolin and 20 μg/ml CM−GGF, as originally described by Porter *et al.* (1986). For each experiment, including the proliferation assays, Schwann cells were cultured in forskolin and GGF for 3−5 weeks, and then withdrawn from these mitogens by washing cell monolayers twice with DMEM and replacing the medium with DMEM plus 10% FBS alone for 4−5 days prior to the start of the experiment. For each induction experiment, forskolin was used at a concentration of 20 μM, although dose−response analyses demonstrated strong induction of PDGF receptor mRNA with drug concentrations as low as 1 μM (data not shown). Forskolin is a fully reversible activator of adenylate cyclase (Seamon *et al.*, 1981). Schwann cells cultured for 3−5 weeks in forskolin and GGF revert to nearly complete quiescence when withdrawn from these mitogens for 4−5 days (Porter *et al.*, 1986; this study).

Proliferation assay

Schwann cell division was measured using a [^{125}I]iododeoxyuridine ([^{125}I]UdR) incorporation assay, as described previously (Brockes *et al.*, 1980; Lemke and Brockes, 1984). 10^4 Schwann cells per microtiter well were used for each of the assay points, which were performed in triplicate. Agents to be tested for proliferative activity were present for a 48 h period, with 2 μCi/ml [^{125}I]UdR being present for the final 24 h. Labeled cells were trypsinized and collected on to glassfiber filters using a multi-sample cell harvester, and incorporated ^{125}I was monitored with a Beckman 5500 gamma counter.

RNA isolation and analysis

Total cellular RNA from cultured cells was isolated according to the guanidine/water-saturated phenol procedure described by Chomczynski and Sacchi (1987). RNA was fractionated on 0.8−1% agarose/formaldehyde gels, and Northern blots were performed according to standard procedures, as described previously (Lemke and Chao, 1988). Following transfer to Nytran® membranes (Schleicher and Schuell), RNA was visualized by staining with methylene blue, as described by Herrin (1988). Briefly, membranes were UV irradiated for 3 min and then treated with 5% glacial acetic acid for 15 min, followed by 0.04% methylene blue in 0.5 M sodium acetate, pH 5.2 for 10 min. Stained membranes were destained with several changes of distilled water over 10 min. For each of the Northern blots presented, underlying methylene blue staining patterns allow for assessment of relative levels of 18S and 28S rRNAs in each lane. All radiolabeled probes were prepared from double-stranded cDNA fragments, using [α-^{32}P]dCTP and a random hexamer priming kit, according to instructions provided by the manufacturer (BRL). Standard high stringency hybridization and washing conditions were used throughout, as described previously (Lemke and Chao, 1988; Monuki *et al.*, 1989).

Western blots

Western immunoblotting was performed as described previously (Weinmaster *et al.*, 1988), with minor modifications. Briefly, cell monolayers on 6 cm dishes were washed twice with Dulbecco's phosphate-buffered saline (Gibco) and lysed by the addition of 200 μl protein sample buffer (5 mM sodium phosphate, pH 6.8, 2% SDS, 0.1 M dithiothreitol, 5% 2-mercaptoethanol, 10% glycerol, 0.4% bromophenol blue) containing 200 μM Na_3VO_4 and 5 mM EDTA. Each sample was boiled for 5 min and equivalent amounts of cellular protein were resolved on an 8% SDS−polyacrylamide gel. Resolved proteins were transferred to Immobilon-P membranes (Millipore), which were then incubated with either anti-phosphotyrosine or anti-PDGF receptor antibodies. Antibody binding was detected by subsequent incubation with 200 μCi of [^{125}I]protein A in 40 ml of blocking buffer, as described previously (Weinmaster *et al.*, 1988). Pre-stained mol. wt markers (Amersham) were electrophoresed on the same gel to aid in the identification of the reactive PDGF receptor protein.

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