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B-50/GAP-43 mRNA expression in cultured primary Schwann cells is regulated by cyclic AMP

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FOLLOWING peripheral nerve crush or transection, B-50 mRNA expression increased dramatically in the distal nerve stump. This increase has been fully attributed to an up-regulation of B-50 synthesis in reactive Schwann cells. Here we describe that B-50 mRNA expression in primary Schwann cell cultures is strongly down-regulated by cyclic AMP. Treatment of neonatal Schwann cell cultures with as low as 20 nM forskolin decreased B-50 mRNA expression. We show that B-50 promoter P2, but not P1, is active in Schwann cells and that the activity of P2 is inhibited 2.5 fold by forskolin. P2 does not contain a consensus sequence of a known cyclic AMP responsive element suggesting that the effect of forskolin is indirect.

Key words: B-50/GAP-43; Schwann cells; Forskolin; Cyclic AMP; Transfection; mRNA; Promoter; Transcription

Introduction

B-50/GAP-43 is a phosphoprotein expressed at high levels during neurite outgrowth and synaptic plasticity.[1,2] Its expression is not restricted to neurones as was believed for many years. Under specific circumstances both *in vivo* and in cell culture, several glial cell types express B-50 protein and mRNA.[3]

We and others have recently demonstrated that peripheral nerve injury results in a dramatic increase in the expression of B-50 mRNA.[4,5] Reactive Schwann cells located in the distal nerve stump express elevated levels of B-50 mRNA[5] and protein.[4] Following regeneration, B-50 mRNA expression is down-regulated to control levels, whereas permanent transection leads to continued high expression. In contrast to the observed increase in the reactive Schwann cells located in the distal nerve stump, no change in B-50 mRNA expression is detectable in the proximal nerve portion. These results suggest that loss of axonal contact induces B-50 mRNA expression in Schwann cells.[5]

Several studies provide evidence that axon-Schwann cell contact plays an important role in the regulation of Schwann cell gene expression. For example, the expression of the major myelin genes P_0 and myelin basic protein (MBP) is sharply down-regulated upon loss of axonal contact. In contrast, Schwann cell expression of the low affinity nerve growth factor (NGF) receptor (p75) mRNA is activated by the withdrawals of axons.[6]

One of the second messengers believed to be implicated in modulating Schwann cell gene expression via axonal contact is cyclic AMP.[6] Primary Schwann cells cultured without axons closely resemble reactive Schwann cells present in the distal never stump after nerve injury. The low expression of the P_0 and MBP genes in these cultured Schwann cells is reversed by addition of forskolin, an agent that elevates intracellular cyclic AMP levels.

The present study was performed to investigate the effect of forskolin on the expression of B-50 mRNA in primary neonatal Schwann cell cultures. In addition, the cultures were transfected with 3 different B-50 promoter-luciferase fusion constructs (containing either P1 or P2, or both) in order to investigate their activity in these primary Schwann cells and to study the effect of forskolin on the transcriptional activity of these promoters.

Material and Methods

Schwann cell culture: Schwann cells were isolated from the sciatic nerves of neonatal (2–3 day) Sprague–Dawley rats and purified by immunoselection as described by Brockes *et al.*[7] Purified cells were cultured at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum and penicillin/streptomycin, with crude glial growth factor and forskolin, as described previously.[6,8]

RNA isolation and analysis: Total RNA was isolated using the guanidine thiocyanate/water-saturated phenol procedure of Chomczynski.[9] RNA samples were denaturated in 50% formamide/2.2 M formaldehyde, electrophoresed in 1% agarose/2.2 M formaldehyde gels, and transferred on to Nytran filters (Schleicher & Schuell). Filters were stained in 0.04% methylene blue/0.5 M, sodium acetate pH 5.2, to visualize ribosomal 28S and 18S bands. Probes for hybridization were prepared using [α-^{32}P]dCTP and a random hexamer priming kit (Gibco-BRL) according to the protocol provided by the manufacturer. In all cases, final wash stringency was set at 0.4 × SSC/1% SDS, 65% C. Prior to reprobing, filters were stripped in 0.01 × SSC, 0.01% SDS at 100°C for 20 min. The following cDNA probes were used: a 1130 bp rat B-50 cDNA isolated from clone pGB$_0$,[10] the complete rat P$_0$ cDNA,[11] a 585 bp SmaI fragment of mouse c-Jun cDNA (gift from Dr I. M. Verma) and a SacII–KpnI rat SCIP cDNA fragment.[8]

Transfection: Transfections were performed using 70–150 × 10^3 cells per 6 cm dish. Four μg of a B-50 promoter-luciferase construct (–1012Luc (1 kb containing P1 and P2), –1012 to –233Luc (P1) and –233Luc(P2)[12]) and 2 μg of the Rous sarcoma virus (RSV) promoter-LacZ construct supplemented with pBluescript to a total of 14 μg DNA was used to transfect each dish. DNAs were precipitated according to standard calcium phosphate procedures[13] and applied to the cells. After 4 h the cells were washed, the medium replaced and cells were cultured for an additional 72 h in the presence or absent of forskolin (Calbiochem). The cells were harvested, cell extracts prepared and the reporter gene enzyme activities were measured according to the procedure described by Eggen *et al.*[12] Several reference and control constructs were included in each transfection series: p19Luc and pLacZ as negative controls, the RSV promoter fused to the luciferase gene (RSVLuc) as positive control and RSVLacZ as an internal reference. Within each experiment transfections were performed in triplicate.

Results

The effect of forskolin, a specific activator of adenylate cyclase, on the expression of B-50 mRNA was investigated in cultured neonatal Schwann cells using Northern blot analysis and transient transfection with B-50 promoter constructs. A low concentration of forskolin (2 μM) was added to the cell cultures and RNA was isolated at various time intervals (Fig. 1). Forskolin completely down-regulated the expression of B-50 mRNA within 12 h (Fig. 1, panel A). Untreated cells still expressed B-50 mRNA after 24 h (see Fig. 2 panel A). The kinetics of the regulation of expression of the B-50 gene following forskolin were compared with other Schwann cell genes known to be regulated by

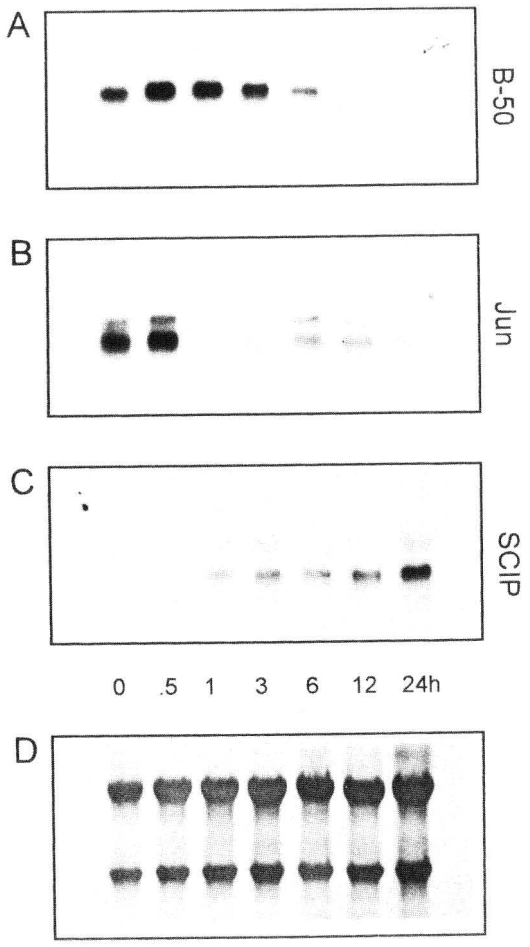

FIG. 1. Effect of forskolin on B-50, c-Jun and SCIP mRNA expression in primary Schwann cell cultures in time. Forskolin (2 μM) was added to the culture and the cells were harvested at the indicated times. RNA (7.5 μg) was separated on a denaturing agarose gel and blotted to Nytran filters. Panels A, B and C show the hybridization with probes for B-50, c-Jun and SCIP respectively. In panel D the amount of RNA in each lane is shown by methylene blue staining for the 28 and 18S ribosomal RNA bands. Note that B-50 mRNA levels gradually decreased after addition of forskolin. In contrast SCIP is upregulated as a result of forskolin administration.

forskolin. The expression of the mRNA for the immediate early transcription factor c-Jun was very rapidly down-regulated (Fig. 1, panel B). In contrast, the effect of forskolin on the expression of SCIP, the POU domain transcription factor, was reciprocal to the effect of forskolin on c-Jun expression (Fig. 1, panel C), which is consistent with previous observations.[8]

The sensitivity of B-50 mRNA expression to forskolin was investigated with a dose-response titration in which RNA from cultured Schwann cells was isolated 24 h after the addition of varying concentrations of forskolin (Fig. 2). Addition of forskolin in a concentration range of 0.2 to 5 μM completely down-regulated B-50 mRNA expression. Concentrations as low as 20 nM forskolin were sufficient to reduce B-50 mRNA expression significantly (Fig. 2, panel A). Concentrations of forskolin required to up-regulate the

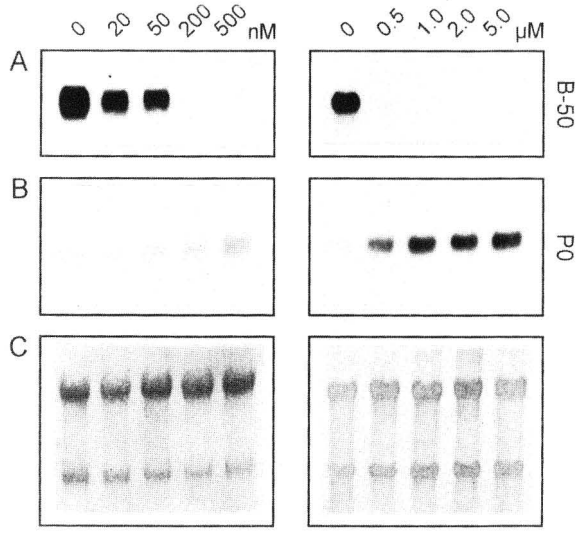

FIG. 2. Sensitivity of B-50 and P_0 mRNA expression to forskolin. Primary Schwann cells were cultured for 24 h with forskolin at the concentrations indicated at the top of panel A. RNA (7.5 μg) was separated on a denaturing agarose gel and blotted to Nytran filters. Panels A and B show the hybridization with probes for B-50 and P_0 respectively. In panel C the amount of RNA in each lane is shown by methylene blue staining for the 28 and 18S ribosomal RNA bands. B-50 mRNA expression is completely suppressed at a concentration of 200 nM forskolin, whereas the expression of P_0 is notably stimulated by 500 nM forskolin.

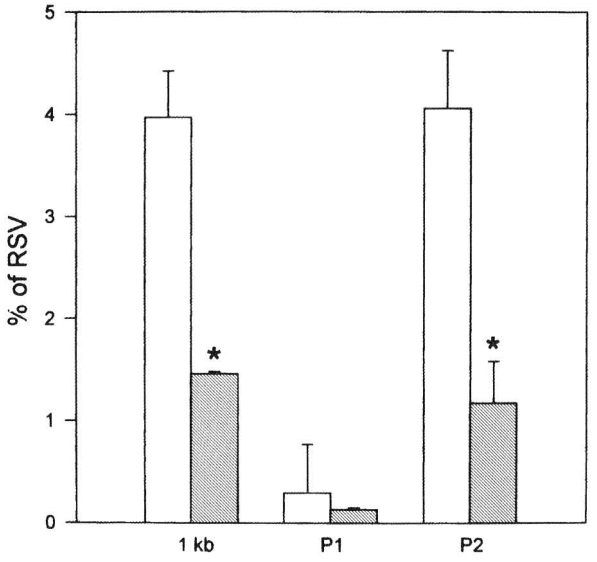

FIG. 3. Effect of forskolin on the activity of the B-50 promoters P1 and P2 using luciferase as the reporter gene. Cells were transfected with the B-50 promoter-luciferase constructs 1kb (–1012Luc], P1 (–1012 to –233Luc) and P2 (–233Luc) and cultured for 72 h in the absence (open bars) or presence (hatched bars) of 4 μM forskolin. The activity of the B-50 promoters is corrected for transfection efficiency and number of cells by co-transfection with RSV-LacZ and expressed as the percentage of the activity of RSV-Luc. Both constructs containing the P2 promoter were active in the primary Schwann cell cultures. The activity of these constructs was decreased by more that 2 fold after treatment of the cells with forskolin. Differences between B-50 promoter activity in the absence and presence of forskolin were evaluated for the 1 kb, the P1 and P2 constructs by means of an unpaired *t*-test ($*p < 0.01$).

expression of a major myelin gene P_0, were at least tenfold higher (Fig. 2, panel B).

To identify regions within the 5′ region of the B-50 gene which can act as a transcription initiation site in neonatal Schwann cell cultures, constructs containing various portions of the upstream B-50 gene fused to the promoterless luciferase gene were analysed in transient transfection assays. The RSV promoter linked to the bacterial LacZ gene was co-transfected to normalize for variations in transfection efficiency and cell number. The RSV promoter linked to the luciferase gene was used as a reference for relative B-50 promoter activity. The 1 kb construct (–1012Luc), containing 1 kb directly upstream of the open reading frame, yielded 4% of the activity of the RSV-Luc construct in the absence of forskolin (Fig. 3, open bars). The P1 construct (–1012 to –233Luc), containing the most 5′ promoter (located between –750 and –405), failed to show significant promoter activity, whereas the P2 construct (233Luc), containing the 3′ promoter (located between –233 and –1), appeared to be as active as the 1 kb construct. Forskolin reduced the activity of both the 1 kb and the P2 promoter construct 2–2.5 fold (Fig. 3, hatched bars) compared with the activity of the RSV-Luc construct.

Discussion and Conclusions

In this report we show that treatment of neonatal Schwann cell cultures with forskolin down-regulates B-50 mRNA expression and upregulates the expression of SCIP and P_0. Transfection of these cells with B-50 promoter constructs containing P1, P2 or both showed that only P2 was active. Moreover it is shown that forskolin inhibits the transcription from the P2 promoter.

In vivo, gene expression in Schwann cells appears to be regulated by axon-Schwann cell contact. In response to axonal loss, fully differentiated myelinating Schwann cells initiate the expression of a set of genes characteristic of the immature Schwann cell. Upon regeneration and restoration of axon-Schwann cell contact, the cells regain their mature phenotype. *In vitro*, it has been demonstrated that Schwann cells cultured in the absence of axons also adopt an immature, dedifferentiated phenotype. Agents that elevate intracellular cyclic AMP, such as forskolin, can to some extent mimic the effect of axons on the pattern of Schwann cell mRNA expression. Lemke *et al*[6] showed that the reduced expression of the myelin-specific proteins P_0 and MBP in cultured Schwann cells is markedly increased after addition of forskolin to the medium. This stimulation of P_0 and MBP gene expressed by forskolin is regulated at the level of gene transcription.[14,15]

Our observation that forskolin down-regulates the high expression of B-50 mRNA in cultured Schwann cells lends further support to the hypothesis that cyclic

AMP is an important second messenger involved in the transition of Schwann cells from an immature to a mature phenotype.

In this report we demonstrate that forskolin mimics the effects of regenerating axons on the B-50 expression in Schwann cells by down-regulating the mRNA levels. In comparison to the effect of forskolin on P_0 mRNA expression, the B-50 gene is down-regulated at cyclic AMP concentrations that are at least 10 times lower than those required to down-regulate P_0. In addition, we show that the effect of forskolin on B-50 mRNA expression is, at least in part, accomplished by inhibition of transcription from promoter P2, the only promoter of B-50 active in primary Schwann cell cultures. In murine P19 embryo carcinoma cells we have previously shown that both promoters are active, but that only the activity of P2 is stimulated by neuroectodermal differentiation.[12] In both human and rat brain poly(A)$^+$ RNA and in SH-SY5Y neuroblastoma cells, transcripts from both promoters can be detected.[12,16,17] The amount of transcripts from P2 however always exceeds that of P1.

So far two different mechanisms that regulate the level of expression of B-50 mRNA are described. NGF stimulates the B-50 expression in PC12 cells by stabilization of the mRNA through a protein kinase C-dependent mechanism,[18-20] whereas glucocorticoids inhibit B-50 transcription *in vitro*.[18] *In vivo*, it has been shown that adrenalectomy increases B-50 mRNA expression in the pyramidal cells of the hippocampus.[21] Whether this *in vivo* effect is caused by a decreased inhibition of B-50 transcription by glucocorticoid is unknown. Both B-50 promoters do not contain steroid responsive binding sites[12,17,22,23] but these sites could be located in yet unidentified intron sequences as suggested by Vanselow *et al.*[24]

Our results show for the first time that the B-50 promoter P2 can be down-regulated by cyclic AMP in primary neonatal Schwann cell cultures. There is however no CREB nor the novel discovered cyclic AMP regulated element of the myelin basic protein promoter present in the P2 sequence.[14] Therefore we believe that the effect of forskolin on the inhibition of P2 transcription is mediated via either an unidentified cyclic AMP responsive element or via cyclic AMP-inducible trans-acting genes.

One of the possible candidates through which cyclic AMP may exert its action is SCIP, a cyclic AMP-inducible transcription factor expressed by Schwann cells. Transcriptional repression of P_0 following forskolin treatment is mediated by SCIP. In the CNS, expression of myelin and B-50 are inversely related and prevention of myelinization increases B-50 expression in the spinal cord.[25] SCIP might act as a transcriptional inhibitor of B-50 gene expression, however, no putative binding site for SCIP has been identified in the P2 promoter.

In conclusion our data show that B-50 gene expression in neonatal Schwann cell cultures is down-regulated by cyclic AMP. Whether this effect is Schwann cell specific or a general mechanism by which B-50 gene expression is down-regulated remains to be investigated. Finally it is interesting to note that B-50 is among the last 'early' Schwann cell genes to come back on folllowing nerve transection, and is the most sensitive to cyclic AMP down-regulation in cultured cells.

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