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Rapid Communication

Modulation of the Neurofibromatosis Type 1 Gene Product, Neurofibromin, During Schwann Cell Differentiation

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Neurofibromin, the product of the neurofibromatosis type 1 (NF1) gene, is a ~250 kDa protein expressed predominantly in cortical neurons and oligodendrocytes in the central nervous system (CNS) and sensory neurons and Schwann cells in the peripheral nervous system (PNS). To gain insight into the biological role of neurofibromin in Schwann cells, the modulation of *NF1* gene expression in a Schwann cell line (MT_4H1) stimulated to either proliferate or differentiate in response to agents that elevate intracellular cAMP was examined. Untreated cells and cells exposed to mitogenic doses of forskolin (1–10 μM) or 8-bromo-cAMP (0.1 mM) expressed low levels of *NF1* mRNA and the protein was barely detectable. High doses of forskolin (100 μM) or 8-bromo-cAMP (1 mM) induced the expression of both myelin P_0 protein and neurofibromin with an identical time course. Although *NF1* mRNA levels peaked within 1–6 hr, the rise in neurofibromin was not apparent until 24–48 hr and peaked 72 hr after treatment. P_0 and neurofibromin were also coinduced by cell-cell contact in high density, untreated cultures. Moreover, differentiation initiated by either cAMP stimulation or high density culture conditions was associated with predominant expression of the type 2 *NF1* mRNA isoform. In contrast, type 1 *NF1* mRNA isoform expression was observed in untreated Schwann cells or those stimulated with mitogenic doses of forskolin or 8-bromo-cAMP. A switch from the type 1 neurofibromin that can efficiently downregulate p21-ras to the type 2 isoform with reduced activity may facilitate a p21-ras signaling pathway associated with Schwann cell differentiation.

Key words: NF1, GTPase-activating protein, p21-ras, cAMP

INTRODUCTION

Von Recklinghausen neurofibromatosis or neurofibromatosis type 1 (NF1) is a common autosomal dominant disorder affecting approximately 1 in 3,500 individuals (Riccardi, 1991). Although there are a variety of different tissue abnormalities, nearly all adult patients with NF1 have neurofibromas (Riccardi, 1981). These are benign tumors which contain predominately Schwann cells (60–85%) with fewer numbers of fibroblasts, perineurial cells, mast cells, pericytes, and vascular elements (Peltonen et al., 1983). When examined in vitro, Schwann cells from neurofibromas were found to have invasive properties and promote angiogenesis indicative of a preneoplastic state (Sheela et al., 1990). In addition, Schwann cells within neurofibromas are more likely to undergo malignant transformation into neurofibrosarcomas (Bader, 1986).

The gene for NF1 encodes an 11–13 kb mRNA expressed in all tissues although in varying amounts (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990; Xu et al., 1990b). Two alternatively spliced mRNA isoforms of *NF1* have been identified and termed type 1 and type 2 (Andersen et al., 1993; Nishi et al., 1991). The type 2 isoform contains an additional 63 base pairs, absent in the type 1 isoform, and is predominantly expressed in differentiated neuronal tissues. The *NF1* gene product, termed neurofibromin, migrates as a ~250 kDa protein on sodium dodecyl sulfate (SDS)-polyacrylamide gels and is expressed in greatest quantities in the nervous system, spleen, and kidney (Daston et al., 1992;

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DeClue et al., 1991; Gutmann et al., 1991). Through comparative sequence analysis, neurofibromin was found to share homology with a family of proteins involved in the regulation of p21-ras (Xu et al., 1990b). These proteins, termed GTPase-activating proteins (GAPs), accelerate the conversion of p21-ras from an active, GTP-bound state to an inactive, GDP-bound state (McCormick, 1989; Wigler, 1990). The isolated GAP-related domain (GRD) of neurofibromin has been shown to function in both yeast and mammalian systems (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990a).

To understand the biological basis for the various tissue abnormalities in NF1, it will be essential to characterize the function of neurofibromin in affected cell types. Schwann cells express relatively high amounts of neurofibromin and appear to be particularly vulnerable to *NF1* gene defects. To investigate the biological role of neurofibromin in Schwann cells, we examined whether changes in Schwann cell function are associated with alterations in *NF1* gene expression. At relatively low doses, agents that elevate intracellular cAMP levels have been shown to stimulate mitosis in cultured Schwann cells, whereas high concentrations induce a differentiated phenotype characterized by increased expression of myelin lipids and proteins (Morgan et al., 1991; Sobue and Pleasure, 1984; Sobue et al., 1986). Using a well-characterized Schwann cell line (Peden et al., 1989, 1990; Tennekoon et al., 1987), we found that differentiation was associated with increased expression of *NF1* mRNA and neurofibromin as well as the predominant expression of the type 2 isoform. This switch in *NF1* isoform expression may facilitate a p21-ras-mediated signal transduction pathway involved in Schwann cell differentiation.

MATERIALS AND METHODS

MT_4H1 Schwann Cell Line

The MT_4H1 Schwann cell line was derived by transfecting primary rat Schwann cells with the SV40 large T antigen under the control of a synthetic, metal-regulated promoter (Peden et al., 1989). To expand the population, cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum, 200 μg/ml G418, and 100 μM $ZnCl_2$. Cells replated in medium without $ZnCl_2$ were treated 5–10 days later when large T antigen is not detectable (Peden et al., 1989). Forskolin (Sigma, St. Louis, MO), dideoxy-forskolin (Calbiochem, La Jolla, CA), 8-bromo-cAMP (Sigma), or diluent alone was added for various intervals of time and at different concentrations. To control for the effects of cell density, MT_4H1 Schwann cells were replated at 1×10^6 cells/100 mm dish (40% confluent) 48 hr before harvest. For some experiments, cells were plated at 2.5×10^6 cells/100 mm dish (100% confluent).

Antibodies and DNA Probes

Myelin P_0 rabbit antiserum was kindly provided by Dr. Bruce Trapp (Johns Hopkins Medical School) and the galactocerebroside (Galc) rabbit antiserum was obtained from Advanced Immunochemicals (Los Angeles, CA). The α-tubulin monoclonal antibody was obtained from Sigma and the neurofibromin G1 antipeptide antibody (residues 1400–1419) has been previously described (Basu et al., 1992). The 1.2 kb P5 probe (nucleotides 6624–7847) was derived from an *NF1* cDNA clone obtained from a cauda equina cDNA library (Wallace et al., 1990).

Growth Rate

Cells were replated in the absence of $ZnCl_2$ and 5–10 days later seeded at 25–30% confluence in 6 or 12 well plates. Low doses of forskolin (1–10 μM) or 8-bromo-cAMP (0.1 mM) were added in fresh feeding medium after the cells had attached. After 24 or 48 hr of treatment, the cells were detached with trypsin and counted in a hemocytometer. Each condition was assayed in triplicate in three separate experiments.

Immunofluorescence

Cells fixed in 4% formaldehyde and permeabilized in 100% methanol for 5 min at −20°C were stained by indirect immunofluorescence (Tennekoon et al., 1987; Gregory et al., 1993). Antibodies were diluted 1:50–1:200 in medium containing 10% serum and photographed using a Zeiss Axiophot microscope with Ektachrome 400 film.

Western Immunoblotting

Cells were rinsed in cold phosphate-buffered saline (PBS) and scrape-harvested in PBS. The cell pellet was lysed in RIPA buffer containing 1% aprotinin, 1% leupeptin, and 1 mM phenylmethylsulfonyl fluoride on ice for 10 min before disruption through a 25 gauge needle. The lysate was then clarified by centrifugation at 14,000 rpm for 15 min at 4°C and the protein concentration of the supernatant was determined using the BioRad protein assay (BioRad Laboratories, Hercules, CA). Protein samples were separated on 7.5% SDS-polyacrylamide gels (100 μg/lane) and transferred to Immobilon (Gutmann et al., 1991). Membranes were incubated overnight at 4°C with antibodies diluted in Tris-buffered saline containing 5% nonfat dry milk and developed using alkaline phosphatase-conjugated secondary antibodies (Gutmann et al., 1991), or with horseradish peroxidase-conjugated secondary antibodies and the ECL detection system (Amersham, Arlington Heights, IL). To measure

neurofibromin levels, the top half of the membrane was incubated with G1 antibodies (1:200) and the bottom half with anti-α-tubulin antibodies (1:1,000) to assess variation in sample loading. Antibodies to P_0 (1:500) were applied to separate membranes.

Northern Blotting

Total RNA was isolated from cells after rinsing the dishes in cold PBS using a modification of the acid-phenol method (RNAzol B; Tel-Test, Inc., Friendswood, TX). RNA concentrations were calculated by ultraviolet (UV) absorption and stored at −70°C in DEPC-treated water. Samples of total RNA (20 μg/lane) were separated on 1.2% agarose-0.37 M formaldehyde gels for 3.5 hr, stained briefly with ethidium bromide, and photographed. RNA was transferred onto nitrocellulose membranes (Hybond, Amersham) and cross-linked by UV exposure (Wallace et al., 1990). The 1.2 kb EcoRI fragment of plasmid P5 and a β-actin cDNA probe were labeled with ^{32}P-dCTP using random hexamers and purified by spin-column chromatography. Membranes were prehybridized in 1× Denhardt's solution (1% SDS/0.2 M dextran sulfate/1 M NaCl) containing 100 μg/ml salmon sperm DNA for 3–12 hr at 65°C, and then hybridized with the probe overnight at 65°C. Membranes were washed in 2× SSC, 0.1% SDS, and exposed to film at −70°C for 2–5 days. Relative changes in *NF1* expression were quantitated by scanning densitometry and the values adjusted to compensate for variations in the amount of total RNA between lanes.

RNA Isoform Analysis

The *NF1* type 1 and type 2 mRNA isoforms were distinguished using primers which span the 63 bp insertion in the GRD (Andersen et al., 1993; Nishi et al., 1991): 5′-GGAACCTCCTTCAGATGACAG-3′ (forward) and 5′-CATGATTGGCAATGCTCTGAA-3′ (reverse). Total RNA (2 μg) was reverse transcribed using Moloney MuLV Superscript reverse transcriptase (Boehringer Mannheim, Indianapolis, IN). The resulting cDNA was amplified by the polymerase chain reaction (PCR) for 30 cycles using a 60°C annealing step (Nishi et al., 1991). The PCR products were analyzed on a 8% polyacrylamide gel stained with ethidium bromide.

RESULTS

Effect of cAMP on Cell Growth Rate

The doubling time of MT_4H1 Schwann cells was 24 hr in the presence of $ZnCl_2$, but increased to 56 hr after maintaining the cells for 5 days without $ZnCl_2$. Treating cells grown in the absence $ZnCl_2$ with low doses of forskolin or cAMP analogs stimulated mitosis with a relatively rapid onset. Within 48 hr, low doses of forskolin (1–10 μM) or 8-bromo-cAMP (0.1 mM) had significantly increased the number of cells per well by 23–54% in 3 separate experiments ($P > 0.05$, Student's t-test).

Effect of cAMP on Cell Phenotype

Treatment of MT_4H1 cells with either 100 μM forskolin or 1 mM 8-bromo-cAMP induced a rapid change in the morphological appearance of the cells which is most pronounced with forskolin (data not shown). The cells initially assumed a more elongated, bipolar shape, but after 48 hr of continued treatment, many cells appeared enlarged and flattened coincident with an increase in myelin P_0 expression. Figure 1 shows MT_4H1 Schwann cells 72 hr after treatment (B) compared to untreated cells (A) stained for P_0 by indirect immunofluorescence. Western immunoblotting demonstrates the change in myelin P_0 levels over time (C). P_0 expression was not altered by treatment with diluent alone, dideoxyforskolin (an analog of forskolin without cAMP-stimulating properties), 0.1 mM 8-bromo-cAMP, or 1–10 μM forskolin (data not shown). Similarly, only the MT_4H1 cells treated with high concentrations of 8-bromo-cAMP or forskolin stained intensely for Galc, another indicator of Schwann cell differentiation (not shown).

Effect of cAMP on *NF1* mRNA Levels

Samples of total RNA from MT_4H1 Schwann cells stimulated with 1–100 μM forskolin for various time intervals were analyzed by Northern blotting with the 1.2 kb P5 probe. The autoradiogram after a 72 hr exposure is shown in Figure 2A, while the ethidium bromide-stained gel before transfer is shown in Figure 2B. Relative changes in *NF1* expression were quantitated by scanning densitometry and the values adjusted to compensate for variations in the amount of total RNA between lanes (Fig. 2C). In addition, probing with the β-actin cDNA probe demonstrated approximately equal loading (data not shown). Northern blot analysis revealed a rapid induction of *NF1* mRNA in response to 100 μM forskolin beginning at 1 hr and remaining elevated for at least 24 hr. Much smaller increases in *NF1* mRNA occurred after treatment with the lower doses. Overexposure of these filters demonstrates low level expression of *NF1* mRNA in untreated MT_4H1 cells. Identical results were obtained on several occasions with forskolin and cAMP analogs (data not shown).

Effect of cAMP on Neurofibromin Expression

No change in neurofibromin expression was detected at 1, 6, or 24 hr after treatment with 1 mM 8-bromo-cAMP even though *NF1* mRNA was markedly increased (data not shown). However, neurofibromin ex-

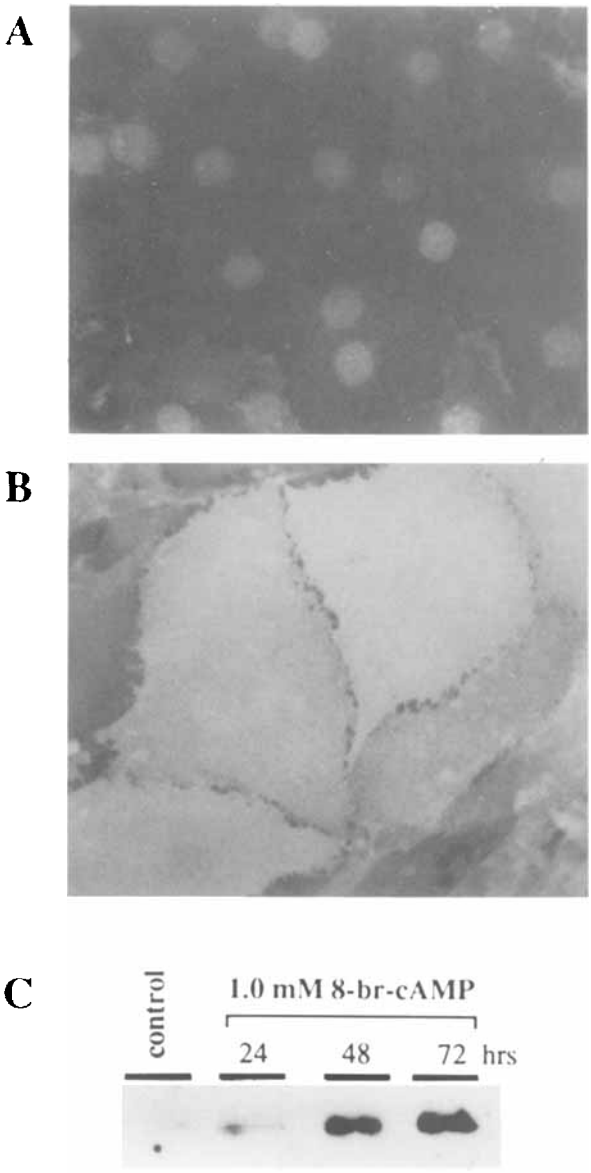

Fig. 1. Expression of myelin P_0 protein in MT_4H1 Schwann cells treated with 1 mM 8-bromo-cAMP. The expression of the myelin P_0 protein was examined in MT_4H1 Schwann cells by indirect immunofluorescence. Untreated cells stained weakly for myelin P_0 protein **(A)**, whereas cells treated with 1 mM 8-bromo-cAMP for 72 hr were brightly positive **(B). C:** The relative change in P_0 expression over time in response to elevating intracellular cAMP was also assessed by Western immunoblotting. The increase in P_0 expression occurred after 24–48 hr of treatment.

pression was induced by 48 hr and the levels peaked 72 hr after treatment (Fig. 3A). Since tubulin levels were not affected, the relative changes in neurofibromin expression over time were adjusted to compensate for variations in loading (Fig. 3B). When compared to Figure 1C, it was apparent that the time course of neurofibromin induction was identical to that of the myelin P_0 protein. Increased neurofibromin expression was also seen with 100 μM forskolin (data not shown); however, low doses of cAMP analogs or forskolin failed to induce the expression of neurofibromin even after 96 hr of stimulation.

NF1 mRNA Isoforms in Differentiating MT_4H1 Schwann Cells

Treatment of MT_4H1 Schwann cells at 40–50% confluence with 1.0 mM 8-bromo-cAMP resulted in predominant expression of the type 2 isoform (Fig. 4, lane 4), whereas cells treated with low doses of untreated cells expressed the type 1 isoform (lanes 2 and 3). The type 2 *NF1* mRNA isoform was also predominant in confluent, untreated cultures (lane 1). Although this PCR analysis is not absolutely quantitative between samples, within a given sample the relative amounts of the two isoforms are accurately reflected. No changes in the relative amount of p120-GAP RNA were detected by PCR in these samples (data not shown). Therefore, differentiation initiated by increasing intracellular cAMP or by cell-cell contact in MT_4H1 Schwann cells is associated with a switch in *NF1* isoform expression from the type 1 to type 2 mRNA isoform.

DISCUSSION

NF1 is a disease in which abnormal Schwann cell growth presumably contributes to the development of neurofibromas. To understand how *NF1* gene defects result in abnormal growth, it will be essential to characterize the biological function of neurofibromin in Schwann cells. In vivo, Schwann cells are normally found only in association with axons which strictly control their function, although the signaling pathways and molecules involved are largely unknown. However, the cAMP pathway has been implicated in the regulation of growth and maturation in several cell types, and agents that increase intracellular cAMP concentration in cultured Schwann cells stimulate either mitosis or expression of the differentiated phenotype, depending on concentration and culture conditions (Morgan et al., 1991; Sobue and Pleasure, 1984; Sobue et al., 1986). To investigate the role of neurofibromin in Schwann cell function, we used the MT_4H1 Schwann cell line which was immortalized with SV40 large T antigen regulated by a synthetic, metal-inducible promoter (Peden et al., 1989). Because T antigen expression is low and tightly regulated, T antigen is not detectable in the absence of the inducer and MT_4H1 cells can fully differentiate to produce myelin (Peden et al., 1990).

We demonstrate here that MT_4H1 cells respond in

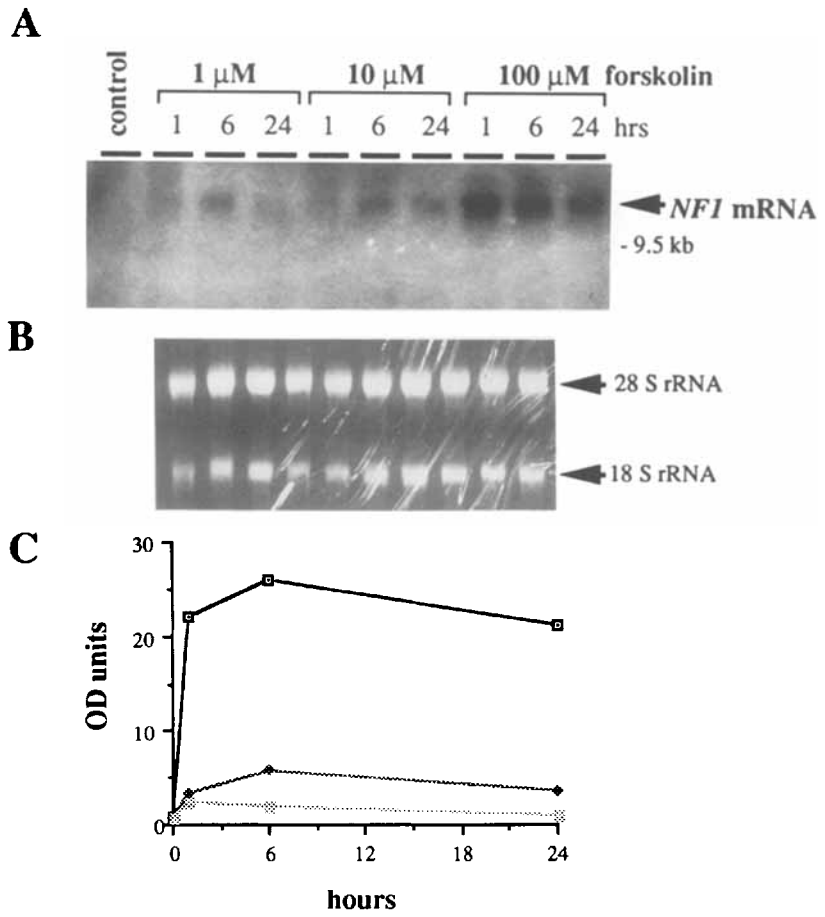

Fig. 2. Differentiating MT_4H1 Schwann cells increase *NF1* mRNA levels. **A:** Northern blot analysis using the P5 cDNA probe demonstrates increased expression of *NF1* mRNA in response to 100 μM forskolin. Much smaller increases were observed with 1–10 μM doses of forskolin. Identical results were obtained with 8-bromo-cAMP stimulation. **B:** Approximately equal loading of RNA is demonstrated by ethidium bromide staining of the gel. **C:** Quantification of the *NF1* mRNA levels relative to the amount of total RNA by scanning densitometry indicated that *NF1* mRNA was rapidly induced and remained elevated for at least 24 hr. Open boxes denote 100 μM forskolin, while the diamonds and closed boxes denote 10 and 1 μM forskolin, respectively.

a similar manner as primary Schwann cells to agents that increase cAMP levels. Low doses of forskolin (1–10 μM) or 8-bromo-cAMP (0.1 mM) were mitogenic (cell numbers increased 23–54% in 48 hr), whereas high doses (100 μM forskolin or 1 mM 8-bromo-cAMP) were necessary to induce the expression of myelin P_0 protein or Galc. Expression of P_0 protein is frequently used as an indicator of the differentiated phenotype which is characterized by coordinated expression of myelin-related components (including P_0, myelin basic protein, and Galc) with repression of glial fibrillary acidic protein, N-cellular adhesion molecule, and the p75 nerve growth factor receptor (Morgan et al., 1991; Sobue and Pleasure, 1984; Sobue et al., 1986). When identical samples were examined for *NF1* gene expression, untreated MT_4H1 Schwann cells and those exposed to mitogenic doses of forskolin or 8-bromo-cAMP were found to express low levels of the type 1 *NF1* mRNA isoform, with barely detectable levels of neurofibromin expression. However, high doses of forskolin or 8-bromo-cAMP induced the expression of neurofibromin with a time course identical to that of myelin P_0 protein. *NF1* mRNA levels peaked within 1–6 hr after treatment, but the rise in neurofibromin was not apparent until 24–48 hr and peaked at 72 hr. The lag in protein induction suggests that neurofibromin expression may be translationally controlled to coordinate with differentiation.

Primary Schwann cells have also been shown to differentiate in growth-arrested, confluent cultures (Morgan et al., 1991), and we found that P_0 and neurofibromin were coinduced in high density, untreated cultures of MT_4H1 cells. Moreover, differentiation initiated by

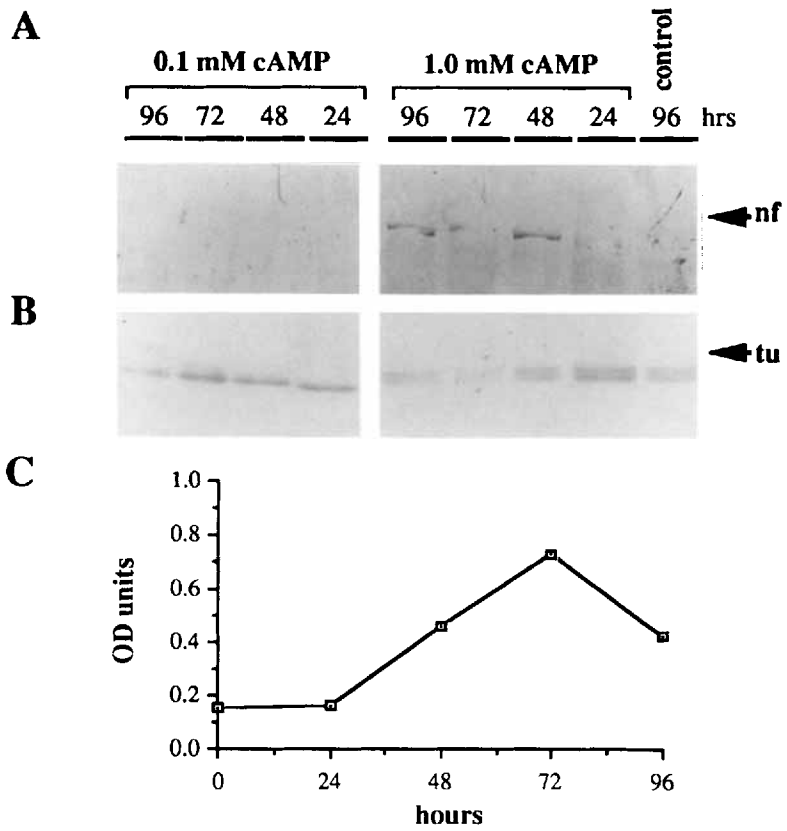

Fig. 3. Differentiating MT_4H1 Schwann cells increase expression of neurofibromin. **A:** Western immunoblotting showed that cells stimulated to differentiate in response to 1 mM 8-bromo-cAMP upregulated neurofibromin (nf) expression after 24–48 hr of treatment. Lower concentrations (0.1 mM) did not alter neurofibromin levels. **B:** To control for variations in protein loading, a portion of the same membrane was incubated with an α-tubulin (tu) monoclonal antibody. **C:** Quantification of neurofibromin levels relative to the amount of tubulin by scanning densitometry indicated that expression peaked 72 hr after treatment.

either elevated cAMP levels or cell-cell contact was correlated with the specific induction of the type 2 isoform of *NF1*. A switch in neurofibromin isoform expression from type 1 to type 2 has previously been reported to occur during neuronal differentiation of neuroblastoma cells (Nishi et al., 1991). The type 2 isoform contains a 63 base pair insertion in the neurofibromin GAP-related domain (Nishi et al., 1991) and has markedly reduced GTPase activity as demonstrated by experiments in yeast and in vitro GAP assays (Andersen et al., 1993). The type 2 GAP-related domain also appears to bind more tightly to p21-ras and conceivably could compete with the type 1 form for p21-ras (Andersen et al., 1993).

Our results suggest the possibility that expression of the type 2 isoform may facilitate a p21-ras signal transduction pathway during Schwann cell differentiation. Introduction of oncogenic p21-ras has previously been shown to inhibit DNA synthesis in Schwann cells, and expression of the v-H-ras causes growth arrest in either the G_1 or G_2/M phase of the cell cycle (Ridley et al., 1988). Growth arrest appears to be a prerequisite for cellular differentiation and induction of the myelin-related phenotype in Schwann cells negatively correlates with mitosis since individual Schwann cells expressing high levels of P_0 do not synthesize DNA (Morgan et al., 1991). It is not known whether activation of p21-ras alters the Schwann cell phenotype; however, activation of p21-ras is sufficient to trigger growth arrest and neuronal differentiation of rat PC12 pheochromocytoma cells (Bar-Sagi and Feramisco, 1985; Noda et al., 1985; Hagag et al., 1986).

Neurofibromin is currently thought to function as a tumor suppressor gene product, and it has been suggested that abnormal growth of Schwann cells in neurofibromas is related to inadequate regulation of p21-ras. In experiments with malignant neurofibrosarcoma cells (presumably Schwann cells), barely detectable levels of neurofibromin and increased levels of activated GTP-

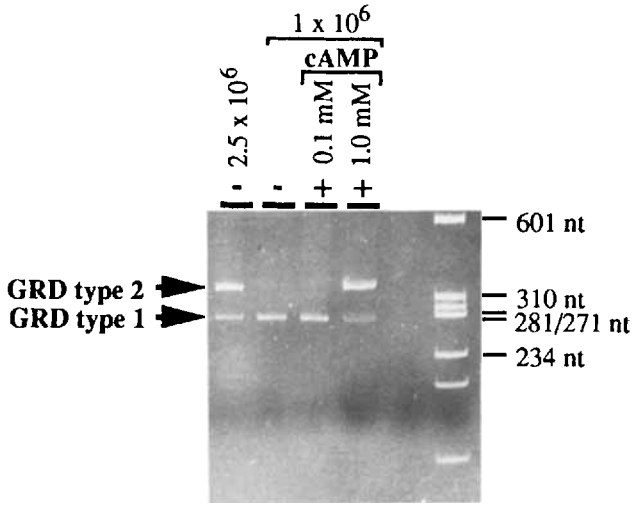

Fig. 4. Isoform analysis of *NF1* mRNA in MT_4H1 Schwann cells. Total RNA was extracted and reverse transcriptase was used to convert mRNA into cDNA. The relative amount of the type 1 and type 2 mRNA isoforms in each sample was determined by PCR. Subconfluent cultures (1.0×10^6 cells/10 cm dish) and cells stimulated with 1.0 mM 8-bromo-cAMP expressed predominantly the type 2 isoform; untreated cells or cells stimulated with 0.1 mM 8-bromo-cAMP expressed the type 1 isoform. Untreated cells in confluent cultures (2.5×10^6/10 cm dish) also expressed more type 2 than type 1.

bound p21-ras were demonstrated (Basu et al., 1992; DeClue et al., 1992). Reintroduction of the GAP-related domain of neurofibromin into these malignant Schwann cells decreased the levels of activated p21-ras and dramatically slowed their growth rate, suggesting that malignant transformation in these cells resulted, at least in part, from elevated levels of activated p21-ras due to the absence of neurofibromin (Basu et al., 1992; DeClue et al., 1992).

Since experiments in nontransformed Schwann cells indicate that p21-ras activation is involved in growth arrest, mutations in the *NF1* gene that affect the ability of neurofibromin to regulate p21-ras would be predicted to block cell growth and would not explain tumor development. However, nuclear oncogenes including c-myc, adenovirus E1A, and SV40 large T antigen act cooperatively with oncogenic H-ras to prevent p21-ras-mediated growth arrest and transform rat Schwann cells (Ridley et al., 1988). The effects of p21-ras may be similarly altered in malignant Schwann cells. Loss of heterozygosity for markers on the short arm of chromosome 17 involving the p53 tumor suppressor gene has been found in several neurofibrosarcomas (Menon et al., 1990), and this and other alterations in combination with neurofibromin loss may play a role in the progression towards malignancy.

Given that the results presented here are consistent with the hypothesis that p21-ras activation is associated with Schwann cell differentiation, how can mutations in the *NF1* gene cause abnormal cell growth if unregulated p21-ras is not entirely responsible? The most likely explanation is that neurofibromin has other functions beside p21-ras regulation. We have found that neurofibromin associates with cytoplasmic microtubules in a number of cell types, including Schwann cells (Gregory et al., 1993; Gutmann et al., 1991a,b). It also appears that tubulin physically interacts with neurofibromin through the GAP-related domain and that this interaction significantly reduces the ability of neurofibromin to regulate p21-ras (Bollag et al., 1993). Given this association, neurofibromin may connect p21-ras regulation with such important microtubule-mediated functions as the control of cell shape. The precise role of neurofibromin and the function of its isoforms in the Schwann cell are likely to be complex. Future studies aimed at determining the interaction between neurofibromin, p21-ras, and microtubules in differentiating Schwann cells should ultimately lead to a better understanding of tumor development in patients with NF1.

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