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Rapid Communication

Modulation of Neurofibromatosis Type 1 Gene Expression During In Vitro Myoblast Differentiation

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Neurofibromin, the protein product of the neurofibromatosis type 1 *(NF1)* gene, has two alternate isoforms which are generated by alternative splicing of two exons. One of these isoforms containing exon 48a is expressed at highest levels in muscle. Since neurofibromin is a p21-ras regulator and has been recently shown to be modulated during Schwann cell differentiation, we examined the expression of the *NF1* gene product during in vitro muscle differentiation. Previous work demonstrated that C_2C_{12} murine myoblast cell differentiation could be blocked by the introduction of an activated p21-ras protein. Using this model system, we demonstrate that differentiating C_2C_{12} cells upregulate the expression of *NF1* mRNA by 2 days of serum starvation concomitant with increased expression of nicotinic acetylcholine receptor mRNA. This upregulation of mRNA expression paralleled an increase in neurofibromin and N-ras levels, but no change in the relative abundance of the isoforms containing exon 23a or exon 48a was observed during in vitro myoblast differentiation. The increase in neurofibromin levels paralleled a decrease in the levels of activated p21-ras as assayed by in vivo ^{32}P-orthophosphate incorporation into p21-ras. These results suggest that in vitro C_2C_{12} cell differentiation is associated with a concomitant increase in *NF1* gene expression and decrease in the proportion of activated p21-ras.

Key words: neurofibromin, muscle, tumor suppressor gene, ras oncogene

INTRODUCTION

Neurofibromatosis type 1 *(NF1)* is a common autosomal dominant disorder that manifests clinically with neurofibromas, cafe-au-lait spots, freckling in non-sun-exposed areas, Lisch nodules, optic pathway gliomas, skeletal abnormalities, and learning disabilities (Riccardi, 1991). The *NF1* gene was identified by positional cloning and its protein product, neurofibromin, was found to be expressed in greatest amounts in brain, peripheral nerve, Schwann cell, oligodendrocyte, adrenal gland, and spleen (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990; DeClue et al., 1991; Gutmann et al., 1991; Daston et al., 1992). Although the function of neurofibromin is largely unknown, it is a member of the GTPase activating protein (GAP) family of proteins which act to regulate p21-ras by accelerating the conversion of the active GTP-bound form of p21-ras to the inactive GDP-bound form (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). This function of neurofibromin may be critical to its function in Schwann cells (Basu et al., 1992; DeClue et al., 1992; Gutmann et al., 1993a).

Recently, we have described the tissue distribution of one of the mRNA isoforms of *NF1*, termed type 3 *NF1* or 3′ALT, resulting from the insertion of an additional 54 nucleotide exon into the extreme carboxyl terminus of the protein (Marchuk et al., 1991; Gutmann et al., 1993b). This type 3 mRNA isoform is predominantly expressed in fetal and adult muscle tissues (Gutmann et al., 1993b). The expression of this isoform of *NF1* in muscle suggested that neurofibromin may have a role in muscle differentiation different from its previously described functions in fibroblasts, Schwann cells, and brain tissue. It had been previously shown that overexpression

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of activated p21-ras (oncogenic p21-ras) resulted in the inhibition of both BC_3H1 and C_2C_{12} muscle cell differentiation (Olson et al., 1987; Payne et al., 1987; Gossett et al., 1988). Given the role of neurofibromin as a regulator of p21-ras in Schwann cells, the relationship between neurofibromin expression, p21-ras activation, and C_2C_{12} cell differentiation was explored. In this report, we demonstrate the coordinate upregulation of neurofibromin expression and downregulation of activated p21-ras during in vitro C_2C_{12} muscle cell differentiation.

MATERIALS AND METHODS

Cell Culture

C_2C_{12} muscle cells were obtained from the ATCC (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum and 100 U/ml penicillin-streptomycin. These cells were passaged every 2–3 days before reaching greater than 80% confluency. Differentiation was initiated by changing the growth medium in cultures with at least 80% confluency to DMEM containing 0.5% fetal bovine serum and 100 U/ml penicillin-streptomycin.

Northern Blot Analysis

Total RNA was extracted from C_2C_{12} cultures appropriately treated using the RNAzol B protocol (Tel-Test, Inc., Friendswood, TX). Twenty micrograms of total RNA was electrophoretically separated on 1.2% agarose gels containing formaldehyde for 3.5–4 hr and transferred overnight in 20× SSC onto Hybond membranes (Amersham, Arlington Heights, IL) as previously described (Gutmann et al., 1993a). Blots were sequentially probed as previously described (Gutmann et al., 1993a) with the 1.2 kb P5 probe (*NF1* gene) and the 950 nt PstI probe from the nicotinic acetylcholine receptor α-subunit gene (nAChR; generously provided by Dr. Dan Goldman, University of Michigan). Blots were stripped in boiling hot 0.1% sodium dodecyl sulfate (SDS) and 0.1× Tris-EDTA for 20–30 min.

Western Blot Analysis

Total protein was extracted from C_2C_{12} cultures appropriately treated in modified RIPA buffer (150 mM NaCl; 10 mM Tris, pH 7.5; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 1 mM EDTA) containing 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and 1% leupeptin as previously described (Gutmann et al., 1993a). Protein concentrations were determined by the BioRad protein assay (New England BioLabs, Beverly, MA) as per the manufacturer's instructions. Five hundred micrograms of total protein was separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto Immobilon membranes. Alternatively, 2–5 mg of total protein was immunoprecipitated using 10 μg of D1 antibody and collected on protein A-Sepharose beads (50% suspension; Pharmacia, Gaithersburgh, MD) before separation by SDS-PAGE. Immunoblotting was performed after blocking the membranes in 5% low fat dry milk-TBST (20 mM Tris, pH 7.5; 150 mM NaCl; 0.05% Tween-20) using 1 μg/ml G1 antibody or the monoclonal p120-ras-GAP and N-ras antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used according to their specifications. Detection was performed using the ECL kit from Amersham.

Isoform Analysis

Two micrograms of total RNA was reverse-transcribed using random hexamers and amplified by the polymerase chain reaction (PCR) using primers specific for the regions flanking exons 23a and 48a (Gutmann et al., 1993a,b). Equal amounts of exon 23a primer set products were separated by 10% PAGE and visualized with ethidium bromide staining (Gutmann et al., 1993a). Equal amounts of exon 48a primer set products were separated by 2% agarose electrophoresis and transferred onto Hybond membranes prior to probing with a ^{32}P-labeled exon 48a oligonucleotide probe. These techniques have previously been described in detail (Gutmann et al., 1993b).

In Vivo p21-ras-GTP Assay

Appropriately treated C_2C_{12} cells were grown in T-25 flasks and phosphate-starved for 2 hr prior to labeling with 250–500 μCi; of ^{32}P-orthophosphate (New England Nuclear, Boston, MA) for 4 hr at 37°C. The monolayer was rinsed twice with cold phosphate buffered saline (PBS), pH 8.0, and then lysed in 500 μliters of lysis buffer (20 mM Tris, pH 7.4; 100 mM NaCl; 20 mM $MgCl_2$; 1% NP-40; 0.5% sodium deoxycholate) containing 1 mM DTT and 1% aprotinin. Lysates were centrifuged for 10 min at 14 K rpm. The supernatant fraction was mixed with 50 μliters of 10% activated charcoal, centrifuged, and that supernatant incubated for 2 hr with agitation with 60 μliters of protein A-Sepharose beads (50% suspension; Pharmacia) precoated with rabbit anti-rat antibodies (Sigma, St. Louis, MO) and 1–2 μg of Y13-259 rat monoclonal antibodies (Oncogene Science, Manhasset, NY). The beads were washed twice in buffer one (10 mM sodium phosphate, pH 7.6; 100 mM NaCl; 20 mM $MgCl_2$; 1 mM EDTA; 1% Triton X-100; 0.5% sodium deoxycholate; 0.1% SDS), twice in buffer two (20 mM Tris, pH 8.3; 250 mM NaCl; 20 mM $MgCl_2$; 1% NP-40; 0.1% SDS), once again in buffer one, and then once in buffer three (10 mM Tris, pH 7.5; 20 mM $MgCl_2$) prior to elution in 10 mM EDTA; 1% SDS; 20 μM GDP; 20 μM GTP at 65°C for

5 min. Samples were separated on PEI chromatographic paper for 2.5 hr in 1.3 M lithium chloride and exposed by autoradiography. Quantitation was performed on a Molecular Dynamics PhosphorImager 400E using Molecular Dynamics ImageQuant software (Sunnyvale, CA).

RESULTS

NF1 mRNA Expression During In Vitro C_2C_{12} Differentiation

The differentiation of C_2C_{12} myoblast cells into myotubes occurs after serum starvation (Schneider and Olson, 1988). Cells grown in medium containing 20% fetal bovine serum (FBS) are abruptly switched to medium containing 0.5% FBS and total RNA harvested at various time points thereafter. Morphological changes are apparent after 2 days in culture but are most pronounced at 3 days and beyond when myotubular structures are evident. Unstimulated cells are kept at <80% confluency as some differentiation is evident when C_2C_{12} cells approach 100% confluency. Total RNA was harvested, electrophoretically separated, and analyzed sequentially with the P5 *NF1* cDNA probe and then the α-subunit of the nAChR probe as a marker of myoblast differentiation. A representative Northern blot is shown in Figure 1. As can be appreciated, there is coordinate upregulation of both the nAChR and *NF1* by day 2 of C_2C_{12} differentiation in vitro. However, the increase in nAChR expression precedes the expression of the *NF1* gene, suggesting that the *NF1* gene is not involved in the early stages of differentiation. Confluent cultures not subjected to serum starvation also demonstrated increased expression of both the nAChR and *NF1* (data not shown). This pattern of expression is similar to the upregulation of the N-ras gene reported by others during C_2C_{12} cell differentiation (Leibovitch et al., 1987). Similar results were also obtained using the BC_3H1 cell line (data not shown).

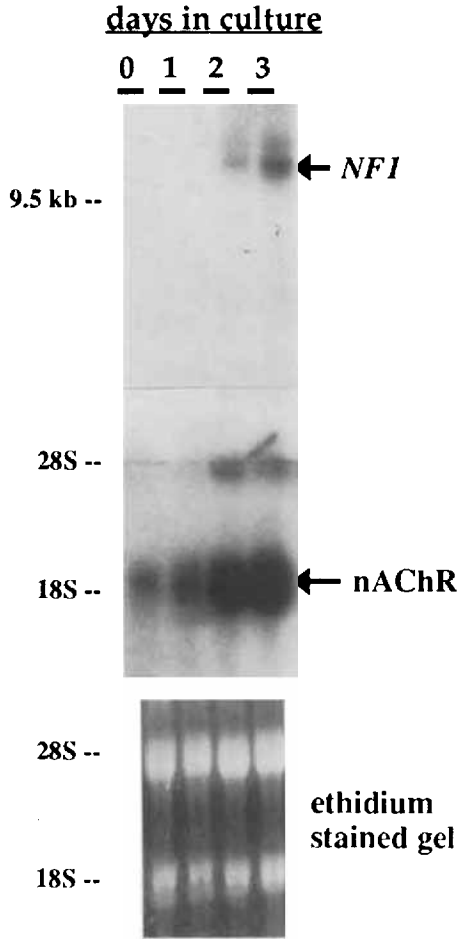

Fig. 1. *NF1* mRNA expression during in vitro C_2C_{12} differentiation. Twenty micrograms of total RNA was separated by agarose electrophoresis, transferred onto Immobilon membranes, and probed sequentially with *NF1* cDNA (P5) and α-subunit of the nAChR cDNA probes as described in Materials and Methods. While low levels of nAChR mRNA are detectable in undifferentiated cells, the expression of *NF1* mRNA and increased expression of nAChR mRNA are seen by 2 days in culture. The ethidium-stained membrane depicts the 28S and 18S RNA bands.

NF1 mRNA Isoform Expression During C_2C_{12} Differentiation

Previously we have shown that during in vitro Schwann cell differentiation, there is a change in the *NF1* isoform expression (Gutmann et al., 1993a). Likewise, during retinoic acid-induced neuroblastoma cell differentiation, a similar change is observed (Nishi et al., 1991). To examine whether a change in *NF1* isoform expression occurred with C_2C_{12} differentiation, total RNA was reverse-transcribed and amplified by PCR using primers flanking the two alternative exons (23a and 48a). As can be seen in Figure 2, there is no change in the expression of type 1 (lacking either insertion), 2 (containing exon 23a), or 3 (containing exon 48a) *NF1* RNA. Likewise, no change in isoform expression was observed with 100% confluent cultures or differentiating BC_3H1 cells (data not shown). No changes in overall expression of *NF1* isoforms are observed since equal amounts of reverse-transcribed PCR products are loaded in each lane.

Neurofibromin Expression During C_2C_{12} Differentiation

To confirm the *NF1* RNA results on the protein level, the expression of neurofibromin was examined during the course of C_2C_{12} differentiation. Total protein extracted from C_2C_{12} cells was either immunoprecipi-

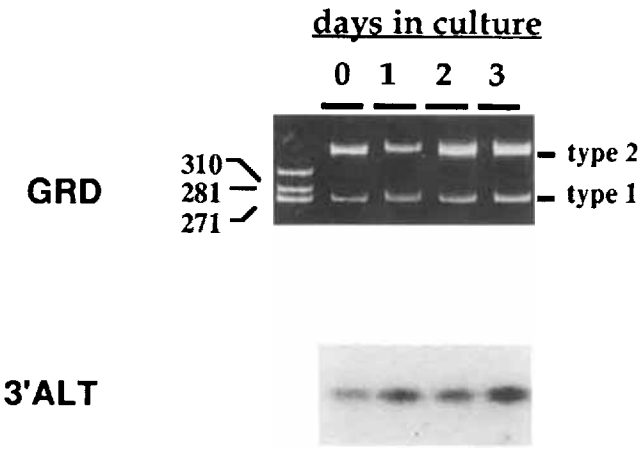

Fig. 2. *NF1* mRNA isoform expression during C_2C_{12} differentiation. Two micrograms of RNA was reverse-transcribed and amplified by PCR as described in Materials and Methods. *NF1* type 1 and type 2 [containing the exon 23a 63 nucleotide insertion into the GAP-related domain (GRD)] PCR products are photographed directly from ethidium bromide-stained 10% polyacrylamide gels. The 3'ALT isoform (containing the exon 48a 54 nucleotide insertion into the extreme carboxyl terminus of the gene) is visualized by separating equivalent amounts of PCR products, transferring overnight to Immobilon membranes, and probing with the 48a exon-specific oligonucleotide.

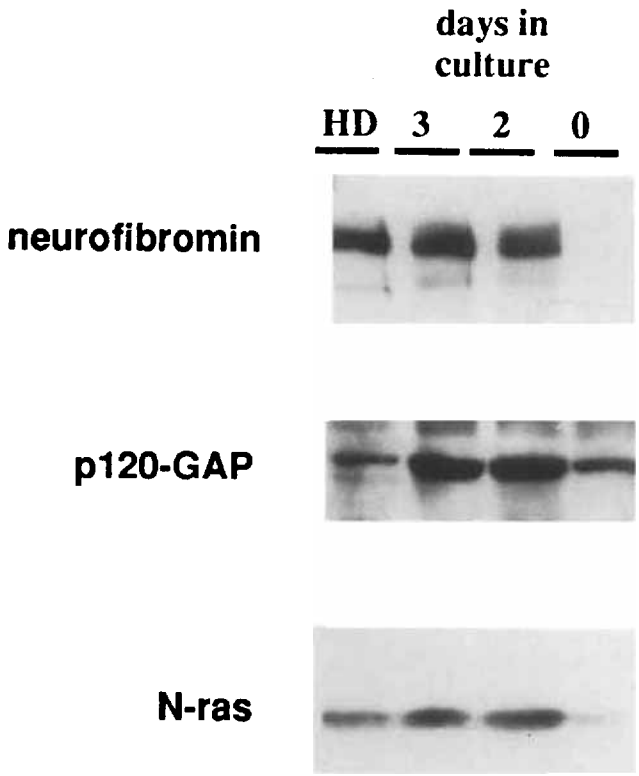

Fig. 3. Neurofibromin expression during C_2C_{12} differentiation. Total protein lysates taken from untreated C_2C_{12} cells, differentiating C_2C_{12} cells, or C_2C_{12} cells grown to 100% confluency for 3 days (HD) were analyzed by immunoblotting. Two milligrams of total lysate was immunoprecipitated with the neurofibromin D1 peptide antiserum for 3–4 hr and collected on protein A-Sepharose beads and separated on 12.5% SDS-polyacrylamide gels along with 500 μg of total lysate for p12-GAP and N-ras immunoblotting. Neurofibromin was detected by immunoblotting with neurofibromin G1 peptide antiserum (1 μg/ml), while N-ras and p120-GAP were detected using monoclonal antisera as described in Materials and Methods.

tated first or simply electrophoretically separated, transferred onto Immobilon membranes, and immunoblotted with neurofibromin and N-ras antibodies. Previously it was shown that N-ras mRNA levels rise during in vitro C_2C_{12} differentiation (Leibovitch et al., 1987). Figure 3 depicts a representative experiment demonstrating the coordinate rise of both neurofibromin and N-ras, but not p120-ras-GAP, during C_2C_{12} differentiation. Increased expression of neurofibromin and N-ras was also seen in 100% confluent C_2C_{12} cultures (labeled HD, Fig. 3), suggesting that cell contact can mimic some of the effects of serum starvation in this cell line. These results were likewise seen on the RNA level (data not shown).

Long-Term Differentiated C_2C_{12} Cultures Continue to Express Neurofibromin

To determine whether the expression of neurofibromin is a transient event during C_2C_{12} differentiation, C_2C_{12} cells were serum-starved for 10–14 days after the morphological appearance of myotubular structures. RNA and protein were extracted and analyzed by Northern and Western immunoblotting. As can be seen in Figure 4, there is continued expression of both *NF1* mRNA and neurofibromin in long-term cultures at higher levels than observed at 3 days. This increased expression in long-term cultures most likely reflects the greater proportion of C_2C_{12} cells in 14 day cultures which have undergone terminal differentiation compared to 3 day cultures. The levels of N-ras and p120-GAP do not reproducibly change during this interval in culture, despite minor variations observed between experiments.

State of p21-ras-GTP During C_2C_{12} Differentiation

Previous work on both C_2C_{12} and BC_3H1 cell lines has demonstrated that differentiation is inhibited by the introduction of activated oncogenic p21-ras (Olson et al., 1987; Payne et al., 1987; Gossett et al., 1988). This suggests that the process of C_2C_{12} cell differentiation may be dependent on down-regulation of p21-ras either by a net increase in total cellular GAP activity or a decrease in total cellular guanosine nucleotide replacing

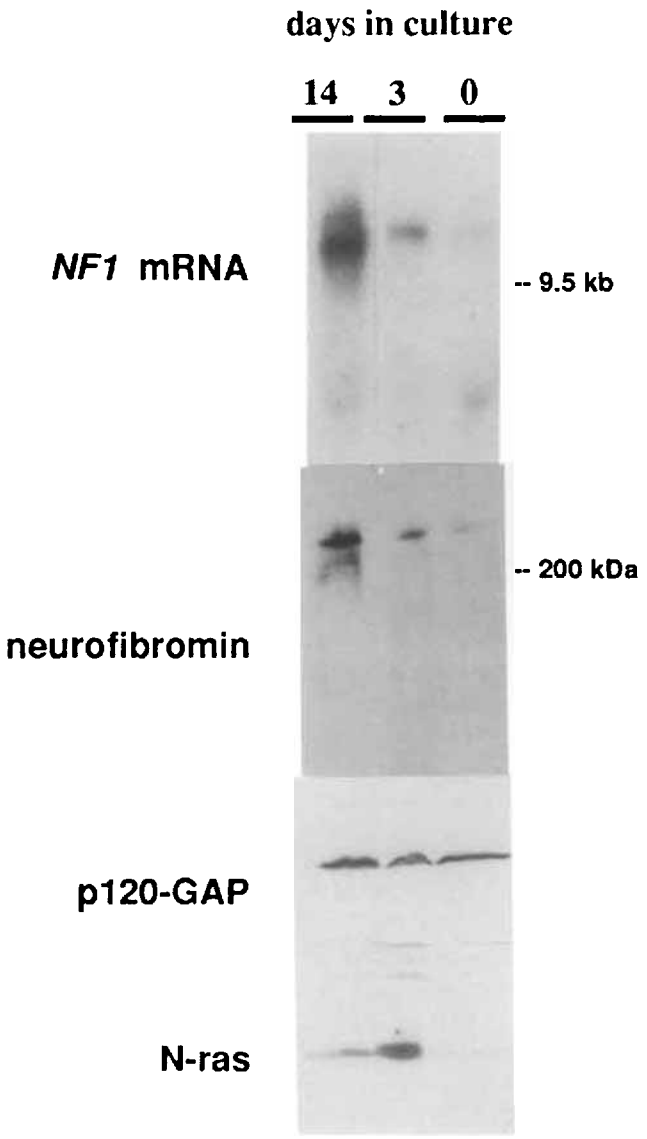

Fig. 4. *NF1* mRNA and neurofibromin expression with prolonged C_2C_{12} cell differentiation. C_2C_{12} cells grown in 0.5% FBS-DMEM for 10–14 days after the appearance of myotubular structures were analyzed by Northern and Western blotting. For Northern blot analysis, 20 μg of total RNA was separated by agarose electrophoresis, transferred onto Immobilon membranes, and probed with *NF1* cDNA (P5) as described in Materials and Methods. For Western immunoblotting, 2 mg of total lysate was immunoprecipitated with the neurofibromin D1 peptide antiserum for 3–4 hr, collected on protein A-Sepharose beads, and separated on 12.5% SDS-polyacrylamide gels along with 500 μg of total lysate for p120-GAP and N-ras immunoblotting. Neurofibromin was detected by immunoblotting with neurofibromin G1 peptide antiserum (1 μg/ml), while N-ras and p120-GAP were detected using monoclonal antisera as described in Materials and Methods.

factor activity. To examine whether the activation state of p21-ras changes during C_2C_{12} differentiation, experiments were undertaken to examine the percent of GTP-bound p21-ras (activated p21-ras) during C_2C_{12} differentiation. The results of three such experiments are represented in Figure 5A. There is a significant decrease in the amount of activated GTP-bound p21-ras in C_2C_{12} cells differentiating for 10 days in culture vs. proliferating, but not confluent, C_2C_{12} cell cultures. Differentiated cultures (3–4 days of differentiation in 0.5% FBS-DMEM) also demonstrate low levels of activated p21-ras (~22% GTP-bound p21-ras; data not shown). In these experiments, there is more p21-ras precipitated from differentiated C_2C_{12} cells owing to the greater cell density in these cultures but the proportion of GTP-bound p21-ras decreases. The levels of GTP-bound p21-ras in our experiments are consistently higher than those observed by others (Basu et al., 1992; DeClue et al., 1992), but remain internally consistent, even with HeLa cell controls (Fig. 5B; data not shown).

DISCUSSION

The successful identification of the gene for *NF1* has opened the door for a more complete understanding of the role of neurofibromin in health and disease. The finding of one *NF1* isoform containing an additional 54 nucleotides (exon 48a) in muscle suggested that neurofibromin may play a unique role in muscle unrelated to the clinical disease. For this reason, an examination of *NF1* gene expression during muscle differentiation was initiated. *NF1* gene and neurofibromin expression are shown to be upregulated during C_2C_{12} cell differentiation in vitro and this increase is paralleled by a decrease in the activation state of p21-ras.

Although the role of neurofibromin in differentiating cells is largely unknown, two properties of neurofibromin are well established. First, neurofibromin is a member of the GAP family of proteins and can accelerate the conversion of p21-ras from the active GTP-bound form to the inactive GDP-bound form (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). Lack of neurofibromin in some cell types is associated with elevated levels of GTP-bound activated p21-ras (Basu et al., 1992; DeClue et al., 1992). Second, neurofibromin associates with cytoplasmic microtubules in some cell types (Gregory et al., 1993). This association of tubulin (a component of microtubules) partially inhibits the ability of neurofibromin to function effectively as a GAP molecule (Bollag et al., 1993).

During cyclic AMP (cAMP)-induced Schwann cell differentiation and retinoic acid-induced neuroblastoma neuronal differentiation, there is a change in *NF1* isoform expression. In these studies, undifferentiated

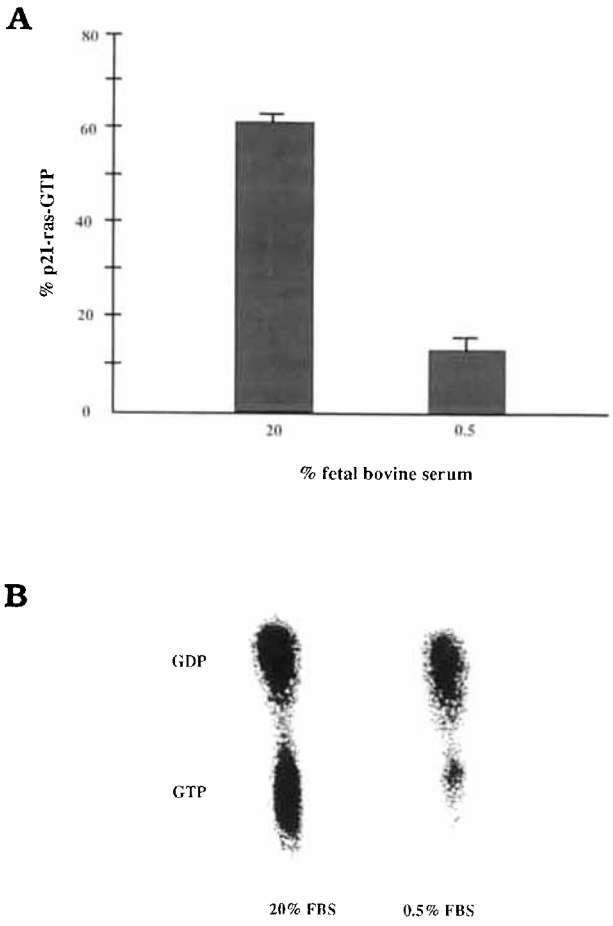

Fig. 5. State of p21-ras-GTP during C_2C_{12} differentiation. In vivo p21-ras-GTP assays were performed on undifferentiated C_2C_{12} cells grown at 75–80% confluency or differentiated C_2C_{12} cells grown for 10–12 days of serum starvation. **A:** Each data point represents the mean and standard deviation for three separate experiments. **B:** PhosphorImage analysis of a representative experiment demonstrating the amount of GTP and GDP eluted from p21-ras immunoprecipitates of undifferentiated and differentiated C_2C_{12} cells resolved by PEI chromatography.

Schwann cells express predominantly type 1 *NF1* (lacking the 63 nucleotide insertion into the GAP-related domain of *NF1*), while differentiating cells express predominantly the type 2 form with this insertion (Gutmann et al., 1993a). Similar results have been obtained using SH-SY5Y neuroblastoma cells in response to retinoic acid stimulation (Nishi et al., 1991). The presence of this insertion in the GAP-related domain of neurofibromin has been shown to dramatically reduce the ability of neurofibromin to function as a GAP molecule (Andersen et al., 1993) and may alter its association with cytoplasmic microtubules (Gutmann et al., manuscript in preparation). The fact that differentiating C_2C_{12} cells do not change their pattern of isoform expression suggests that changes in neurofibromin function are entirely due to an increase in total neurofibromin.

The observation that neurofibromin expression is dramatically increased during C_2C_{12} cell differentiation suggests that the *NF1* gene product may be part of this differentiation program. Previous studies on C_2C_{12} and BC_3H1 cell lines have demonstrated that overexpression of activated p21-ras (N-ras or H-ras) blocks myoblast differentiation (Olson et al., 1987; Payne et al., 1987; Gossett et al., 1988) by inhibiting MyoD1 and myogenin expression through transcriptional regulation (Lassar et al., 1989). However, the relationship between increased neurofibromin expression, decreased p21-ras activation, and C_2C_{12} muscle cell differentiation may be more complicated. Three possibilities can be envisioned. One model has increased neurofibromin expression and decreased levels of activated p21-ras as *unrelated events* common to the C_2C_{12} differentiation program. This implies that the decrease in GTP-bound p21-ras is not related to an overexpression of neurofibromin, but rather to another event. Similarly, it is possible that p21-ras through its interactions with other proteins actually regulates *NF1* gene expression. In yeast cells, RAS activates adenylate cyclase and results in an increase in cAMP (Broach, 1991). In Schwann cells, increasing the levels of intracellular cAMP is associated with increased neurofibromin expression (Gutmann et al., 1993a). This increased *NF1* gene expression may therefore be related to the presence of a putative cAMP-responsive element in the 5′-untranslated region of the *NF1* gene (Hajra et al., manuscript in preparation).

A second model envisions that the increase in neurofibromin expression *results* in a decrease in activated GTP-bound p21-ras by accelerating the conversion of p21-ras-GTP to p21-ras-GDP. This is a tantalizing notion which will need to be addressed in future studies aimed at determining the effect of neurofibromin overexpression on C_2C_{12} differentiation and p21-ras-GTP levels. To demonstrate a causal relationship between neurofibromin expression, p21-ras activation, and C_2C_{12} cell differentiation, we attempted to examine the effects of upregulating and downregulating *NF1* gene expression. Experiments in which the GAP regulatory domain of neurofibromin (NF1GRD) was stably transfected into C_2C_{12} cells failed to yield clones overexpressing neurofibromin yet were clearly resistant to G418 (neomycin): In two experiments, 20 individual G418-resistant colonies and in two other experiments, two pools of 40 G418-resistant colonies each were examined for NF1GRD expression. None of these samples expressed NF1GRD protein. It is possible that this failure reflects activation of the C_2C_{12} cell differentiation program and loss of these neurofibromin-expressing cells from the dividing

G418-resistant population. The construction of a suitable inducible expression vector is now in progress to examine this possibility. Likewise, efforts at downregulating neurofibromin expression using phosphorothioate antisense and control sense oligonucleotides were unsuccessful at modulating *NF1* expression. It is most likely that neurofibromin expression is related to the C_2C_{12} cell differentiation program, but is not required for its initiation or early commitment.

A third model envisions that the increase in neurofibromin and reduction of activated p21-ras are related to *cell density effects* and not differentiation. This possibility, although unlikely given the activated p21-ras experiments, cannot be excluded at this time. The examination of non-fusing variants for changes in neurofibromin levels and p21-ras activation under high and low density conditions as well as the effects of transforming growth factor beta (TGF-β) on the expression of neurofibromin will help to address this issue.

Previous work by a number of laboratories have demonstrated that there is little neurofibromin expressed in adult cardiac and skeletal muscle (Daston et al., 1992; Golubic et al., 1992). However, there is expression of neurofibromin in the developing heart and myotomes of chicks and mice (Daston and Ratner, 1992; Barald, personal communication, 1993). This expression is lost later during fetal development. Mice made homozygously deficient in *NF1* gene expression by targeted gene disruption develop cardiac developmental defects (Shih, Jacks, and Weinberg, personal communication). These observations suggest that neurofibromin expression might be important in muscle development. Future studies directed at examining the role of neurofibromin during in vivo muscle differentiation and regeneration will provide invaluable insights into the function of this tumor suppressor gene product.

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REFERENCES

Andersen LB, Ballester R, Marchuk DA, Chang E, Gutmann DH, Saulino AM, Camonis J, Wigler M, Collins FS (1993): A conserved alternative splice in the von Recklinghausen neurofibromatosis *(NF1)* gene produces two neurofibromin isoforms, both of which have GTPase activating protein activity. Mol Cell Biol 13:487–495.

Ballester R, Marchuk DA, Boguski M, Saulino AM, Letcher R, Wigler M, Collins FS (1990): The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell 63:851–859.

Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS, Downward J (1992): Aberrant regulation of ras proteins in tumour cells from type 1 neurofibromatosis patients. Nature 356:713–715.

Bollag G, McCormick F, Clark R (1993): Characterization of full-length neurofibromin: Tubulin inhibits Ras GAP activity. EMBO J 12:1923–1927.

Broach JR (1991): RAS genes in *Saccharomyces cerevisiae:* Signal transduction in search of a pathway. Trends Genet 7:28–33.

Cawthon RM, Weiss M, Xu G, Viskochil D, Culver M, Stevens J, Robertson M, Dunn D, Gesteland R, O'Connell P, White R (1990): A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell 62:193–201.

Daston MM, Ratner N (1993): Neurofibromin, a predominantly neuronal GTPase activating protein in the adult, is ubiquitously expressed during development. Dev Dyn 195:216–226.

Daston MM, Scrable H, Norlund M, Sturbaum AK, Nissen LM, Ratner N (1992): The protein product of the neurofibromatosis type 1 gene is expressed at highest abundance in neurons, Schwann cells and oligodendrocytes. Neuron 8:415–428.

DeClue JE, Cohen BD, Lowry DR (1991): Identification and characterization of the neurofibromatosis type 1 gene product. Proc Natl Acad Sci USA 88:9914–9918.

DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC, Lowry DR (1992): Abnormal regulation of mammalian $p21^{ras}$ contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 69:265–273.

Golubic M, Roudebush M, Dobrowolski S, Wolfman A, Stacey DW (1992): Catalytic properties, tissue, and intracellular distribution of the native neurofibromatosis type 1 protein. Oncogene 7:2151–2159.

Gossett LA, Zhang W, Olson EN (1988): Dexamethasone-dependent inhibition of differentiation of C2 myoblasts bearing steroid-inducible N-ras oncogenes. J Cell Biol 106:2127–2137.

Gregory PE, Gutmann DH, Boguski M, Mitchell AM, Parks S, Jacks T, Wood DL, Jove R, Collins FS (1993): The neurofibromatosis type 1 gene product, neurofibromin, associates with microtubules. Somatic Cell Mol Genet 19:265–274.

Gutmann DH, Wood DL, Collins FS (1991): Identification of the neurofibromatosis type 1 gene product. Proc Natl Acad Sci USA 88:9658–9662.

Gutmann DH, Tennekoon GI, Cole JL, Collins FS, Rutkowski JL (1993a): Modulation of the neurofibromatosis type 1 gene product, neurofibromin, during Schwann cell differentiation. J Neurosci Res 36:216–223.

Gutmann DH, Andersen LB, Cole JL, Swaroop M, Collins FS (1993b): An alternatively spliced mRNA in the carboxy terminus of the neurofibromatosis type 1 *(NF1)* gene is expressed in muscle. Hum Mol Genet 2:989–992.

Lassar AB, Thayer MJ, Overell RW, Weintraub H (1989): Transformation by activated ras or fos prevents myogenesis by inhibiting expression of MyoD1. Cell 58:659–667.

Leibovitch M-P, Leibovitch SA, Hillion J, Guillier M, Schmitz A, Harel J (1987): Possible role of c-fos, c-N-ras and c-mos proto-oncogenes in muscular development. Exp Cell Res 170:80–92.

Marchuk DA, Saulino AM, Tavakkol R, Swaroop M, Wallace MR,

Andersen LB, Mitchell AL, Gutmann DH, Boguski M, Collins FS (1991): cDNA cloning of the type 1 neurofibromatosis gene: Complete sequence of the *NF1* gene product. Genomics 11: 931–940.

Martin GA, Viskochil D, Bollag G, McCabe PC, Crosier WJ, Haubruck H, Conroy L, Clark R, O'Connell P, Cawthon RM, Innis MA, McCormick F (1990): The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell 63:843–849.

Nishi T, Lee PSY, Oka K, Levin VA, Tanase S, Morino Y, Saya H (1991): Differential expression of two types of the neurofibromatosis type 1 *(NF1)* gene transcripts related to neuronal differentiation. Oncogene 6:1555–1559.

Olson EN, Spizz G, Tainsky MA (1987): The oncogenic forms of N-ras or H-ras prevent skeletal myoblast differentiation. Mol Cell Biol 7:2104–2111.

Payne PA, Olson EN, Hsiau P, Roberts R, Perryman MB, Schneider MD (1987): An activated c-Ha-ras allele blocks the induction of muscle-specific genes whose expression is contingent on mitogen withdrawl. Proc Natl Acad Sci USA 84:8956–8960.

Riccardi VM (1991): Neurofibromatosis: Past, present and future. N Engl J Med 324:1283–1285.

Schneider MD, Olson EN (1988): Control of myogenic differentiation by cellular oncogenes. Mol Neurobiol 2:1–39.

Viskochil D, Buchberg AM, Xu G, Cawthorn RM, Stevens J, Wolff RK, Culver M, Carey JC, Copeland NG, Jenkins NA, White R, O'Connell P (1990): Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell 62: 187–192.

Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulino AM, Fountain JW, Brereton A, Nicholson J, Mitchell AL, Brownstein BH, Collins FS (1990): Type 1 neurofibromatosis gene: Identification of a large transcript disrupted in three *NF1* patients. Science 249:181–186.

Xu G, Lin B, Tanaka K, Dunn D, Wood D, Gesteland R, White R, Weiss R, Tamanoi F (1990): The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of *S. cerevisiae*. Cell 63:835–841.