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 C2C12 murine myoblast cell differentiation could be blocked by the introduction of an activated p21-ras protein. Using this model system, we demonstrate that differentiating C2C12 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 32P-orthophosphate incorporation into p21-ras. These results suggest that in vitro C2C12 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...
of activated p21-ras (oncogenic p21-ras) resulted in the inhibition of both BC3H1 and C2C12 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 C2C12 cell differentiation was explored. In this report, we demonstrate the coordinate upregulation of neurofibromin expression and downregulation of activated p21-ras during in vitro C2C12 muscle cell differentiation.

MATERIALS AND METHODS

Cell Culture

C2C12 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 C2C12 cultures appropriately treated using the RNAzol B protocol (TelTest, 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 20X SSC onto Hybond membranes prior to probing with 250-500 pCi of 3ZP-orthophosphate (New England Nuclear, Boston, MA) for 4 hr at 37°C. The membranes were then washed twice in 0.05% Tween-20) using 1 pg/ml G1 antibody or the 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 C2C12 cells were grown in T-25 flasks and phosphate-starved for 2 hr prior to labeling with 250–500 μCi; of 32P-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 MgCl2; 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) pre-coated 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 MgCl2; 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 MgCl2; 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 MgCl2) prior to elution in 10 mM EDTA; 1% SDS; 20 μM GDP; 20 μM GTP at 65°C for

transferred overnight in 20X SSC onto Hybond membranes prior to probing with 250-500 pCi of 3ZP-orthophosphate (New England Nuclear, Boston, MA) for 4 hr at 37°C. The membranes were then washed twice in 0.05% Tween-20) using 1 pg/ml G1 antibody or the labeled exon 48a oligonucleotide probe. These techniques have previously been described in detail (Gutmann et al., 1993b).

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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 C2C12 Differentiation

The differentiation of C2C12 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 C2C12 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 C2C12 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 C2C12 cell differentiation (Leibovitch et al., 1987). Similar results were also obtained using the BC3H1 cell line (data not shown).

NF1 mRNA Isoform Expression During C2C12 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 C2C12 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 BC3H1 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 C2C12 Differentiation

To confirm the NF1 RNA results on the protein level, the expression of neurofibromin was examined during the course of C2C12 differentiation. Total protein extracted from C2C12 cells was either immunoprecipi-
NF1 Gene Expression in Muscle Differentiation

Fig. 2. *NF1* mRNA isoform expression during C2C12 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 C2C12 differentiation. Total protein lysates taken from untreated C2C12 cells, differentiating C2C12 cells, or C2C12 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 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.

Long-Term Differentiated C2C12 Cultures Continue to Express Neurofibromin

To determine whether the expression of neurofibromin is a transient event during C2C12 differentiation, C2C12 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 C2C12 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 C2C12 Differentiation

Previous work on both C2C12 and BC3H1 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 C2C12 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 C2C12 cell differentiation. C2C12 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.

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 C2C12 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
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\textsubscript{2}C\textsubscript{12} cell differentiation suggests that the N\textit{F1} gene product may be part of this differentiation program. Previous studies on C\textsubscript{2}C\textsubscript{12} and BC\textsubscript{3}H1 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\textsubscript{2}C\textsubscript{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\textsubscript{2}C\textsubscript{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 N\textit{F1} 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 N\textit{F1} gene expression may therefore be related to the presence of a putative cAMP-responsive element in the 5'-untranslated region of the N\textit{F1} 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\textsubscript{2}C\textsubscript{12} differentiation and p21-ras-GTP levels. To demonstrate a causal relationship between neurofibromin expression, p21-ras activation, and C\textsubscript{2}C\textsubscript{12} cell differentiation, we attempted to examine the effects of upregulating and downregulating N\textit{F1} gene expression. Experiments in which the GAP regulatory domain of neurofibromin (N\textit{F1GRD}) was stably transfected into C\textsubscript{2}C\textsubscript{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 N\textit{F1GRD} expression. None of these samples expressed N\textit{F1GRD} protein. It is possible that this failure reflects activation of the C\textsubscript{2}C\textsubscript{12} cell differentiation program and loss of these neurofibromin-expressing cells from the dividing

Schwann cells express predominantly type 1 N\textit{F1} (lacking the 63 nucleotide insertion into the GAP-related domain of N\textit{F1}), 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\textsubscript{2}C\textsubscript{12} cells do not
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 \textit{NFI} expression. It is most likely that neurofibromin expression is related to the C2C12 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-\beta) 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 \textit{NFI} 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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