Dexamethasone-dependent Inhibition of Differentiation of C2 Myoblasts Bearing Steroid-inducible N-ras Oncogenes

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Abstract. ras proteins are localized to the plasma membrane where they are postulated to interact with growth factor receptors and other proximal elements in intracellular cascades triggered by growth factors. The molecular events associated with terminal differentiation of certain skeletal myoblasts are inhibited by specific polypeptide growth factors and by constitutive expression of transforming ras oncogenes. To determine whether the inhibitory effects of ras on myogenic differentiation were reversible and to investigate whether muscle-specific genes remained susceptible to ras-dependent repression in terminally differentiated myotubes, the murine myoblast cell line, C2, was transfected with a plasmid containing a mutationally activated human N-ras oncogene under transcriptional control of the steroid-sensitive promoter of the mouse mammary tumor virus long terminal repeat. Addition of dexamethasone to myoblasts bearing steroid-inducible ras oncogenes prevented myotube formation and induction of muscle creatine kinase and acetylcholine receptors. inhibition of differentiation by dexamethasone occurred in a dose-dependent manner and was a titratable function of ras expression. In the presence of dexamethasone, myoblasts bearing steroid-inducible ras genes retained their dependence on exogenous growth factors to divide and exhibited contact inhibition of growth at confluent densities, indicating that the inhibitory effects of ras on differentiation were independent of cell proliferation. Removal of dexamethasone from N-ras-transfected myoblasts led to fusion and induction of muscle-specific gene products in a manner indistinguishable from control C2 cells. Examination of the effects of culture media conditioned by ras-transfected myoblasts on differentiation of normal C2 cells yielded no evidence for inhibition of differentiation via an autocrine mechanism. In contrast to the ability of N-ras to prevent up-regulation of muscle-specific gene products in myoblasts, induction of N-ras in terminally differentiated myotubes failed to extinguish muscle-specific gene expression. Together, these results suggest that oncogenic ras proteins reversibly activate an intracellular cascade that prevents establishment of the differentiated phenotype. The inability of ras to extinguish muscle-specific gene expression in terminally differentiated myotubes also suggests that ras may interfere with an early step in the pathway of myoblasts toward the differentiated state.

The process of myogenic differentiation involves an ordered sequence of molecular events which include cessation of cell division, formation of multinucleated myotubes, and coordinate induction of a battery of muscle-specific genes encoding proteins involved in the specialized functions of the myofiber (for review, see reference 7). Among the gene products that are up-regulated during myogenesis are the components of the contractile apparatus, the muscle isozyme of creatine kinase (MCK), and the nicotinic acetylcholine (ACh) receptor. The ability of certain skeletal myoblasts to differentiate is controlled in a negative manner by the extracellular concentration of serum mitogens, fibroblast growth factor (FGF) and type β transforming growth factor (TGFβ) (6, 14, 18, 28, 29, 32, 33, 38-40, 52, 53). In the presence of these growth factors, myoblasts fail to fuse or to express muscle-specific gene products. Conversely, reduction in the concentration of these growth factors below a critical level results in irreversible withdrawal from the cell cycle and terminal differentiation. After fusion, muscle-specific genes become refractory to the inhibitory effects of polypeptide growth factors. Little is known of the intracellular pathways whereby polypeptide growth factors influence the muscle differentiation program or of the mechanisms responsible for the loss of sensitivity of muscle-specific genes in myotubes to growth factors.

The products of proto-oncogenes have been postulated to play important roles in regulation of cellular proliferation and

1. Abbreviations used in this paper: ACh, acetylcholine; CK, creatine kinase; FGF, fibroblast growth factor; HS, horse serum; M, muscle isoenzyme; MMTV-LTR, mouse mammary tumor virus long terminal repeat; TGFβ, type β transforming growth factor.
differentiation by transducing growth factor signals from the cell surface to the nucleus (3, 22, 50). As an initial step toward understanding the mechanisms involved in growth factor-mediated regulation of myogenesis, we have chosen to examine the consequences of autonomous expression of specific oncogenes on myogenic differentiation. Recently, Schneider et al. reported that deregulated expression of c-myc, the putative intranuclear mediator of growth factor signals, partially inhibited but did not prevent, induction of muscle-specific genes in the BC3H1 muscle cell line (49). In contrast, mutationally activated ras oncogenes conferred a phenotype on myoblasts similar to that elicited by FGF and TGFβ and suppressed completely the ability of myoblasts to differentiate (41).

The mammalian ras family consists of the Harvey (H)-ras, Kirsten (K)-ras, and N-ras genes, each of which encodes a 21-kD GTP-binding proteins localized to the cytoplasmic surface of the plasma membrane (for review, see reference 30). Ras proteins appear to participate in transduction of mitogenic signals by interacting with specific cell surface growth factor receptors and other proximal elements in cascades triggered by growth factors (17, 27, 30, 37, 55, 59). Termination of transduced signals between receptors and enzyme effectors is postulated to be achieved through hydrolysis of GTP by endogenous GTPase activity of normal ras proteins. ras genes bearing point mutations, generally at codons 12 or 61, have been isolated from a wide range of tumors and have been shown to transform human and rodent cells (4, 30, 45, 57, 58). These mutations do not influence the subcellular localization of the proteins or their ability to bind guanine nucleotides, but in most cases result in diminished GTPase activity (12, 16, 34, 56). The loss of GTPase activity of mutant ras proteins has been associated with persistent activation of specific intracellular growth factor cascades and alleviation of growth factor requirements.

Previously, we reported that constitutive expression of the oncogenic forms of N- and H-ras prevented myoblast differentiation (41). Several important questions concerning the mechanism for ras-dependent inhibition of myogenesis, however, were not amenable to experimental analysis in differentiation-defective cell lines that expressed ras constitutively. For example, it remained unclear whether the inhibitory effects of ras on differentiation were reversible, and if so, whether the kinetics for induction of muscle-specific genes would be accelerated or delayed after release of myoblasts from ras-dependent repression. It was also unknown whether muscle-specific genes were susceptible to ras only during a specific period early in the differentiation program or whether these genes remained responsive to ras in terminally differentiated myotubes. The latter issue was of particular interest in light of observations that muscle-specific genes no longer respond to serum mitogens, FGF or TGFβ after myoblast fusion (6, 14, 32, 33, 40).

Here, we report that transfection of the C2 myoblast cell line with a mutationally activated N-ras gene linked to the steroid-inducible mouse mammary tumor virus long terminal repeat (MMTV-LTR), renders myoblasts sensitive to inhibition of differentiation by dexamethasone. Suppression of the differentiated state by dexamethasone occurred in a dose-dependent manner and required the continual presence of the steroid in the culture medium. Removal of dexamethasone led to fusion and induction of muscle-specific gene products with kinetics indistinguishable from control C2 myoblasts. The mechanism whereby ras inhibited differentiation did not require cell proliferation and was apparently not mediated by autocrine growth factors. In contrast to the ability of ras to prevent differentiation of myoblasts, induction of ras in terminally differentiated myotubes was without effect on myotube morphology or expression of muscle-specific genes. Together, these results indicate that ras inhibits myogenic differentiation through a highly transient and reversible mechanism and that this ras-dependent pathway interferes with an early step in the process of muscle-specific gene activation. In each of these respects, the mechanism for suppression of differentiation by ras resembles the mechanism used by TGFβ and FGF (6, 14, 32, 33, 40).

**Materials and Methods**

**Cell Culture**

The mouse skeletal muscle cell line, C2 (60) was grown in DME containing 20% FCS, as described (40). To initiate differentiation, cultures at ~80% confluence were transferred to DME containing 10% horse serum (HS). Dexamethasone (Sigma Chemical Co., St. Louis, MO) was prepared as a 0.1 mM stock in sterile deionized water and was added to culture media at the concentrations indicated. To obtain pure cultures of myotubes, confluent cultures that had been exposed to DME with 10% HS for at least 48 h were transferred to DME with 20% FCS and 0.1 mM cytosine arabinoside for 48 h. Aphidicolin (Sigma Chemical Co.) was stored as a 1 mg/ml stock in 100% ethanol and was added to a final concentration of 5 μg/ml to DME containing 10% HS as an alternative method of obtaining pure myotube cultures. Cell numbers were determined with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL).

In experiments that required coculturing of C2 cells with CO25 cells, barriers of silicon grease ~2-mm wide were applied to the centers of 35-mm culture dishes. Cells of each type were plated at equivalent densities on opposite sides of the barrier, which was of sufficient height to prevent exchange of media between the two sides. After cells had attached to the substrate, residual nonadherent cells were removed, the barrier was aspirated away such that only a thin line of silicon grease remained on the dish to separate the two cell types, and fresh medium with or without dexamethasone, as specified, was added to the cultures. Cultures were then placed on a rotating shaker at 60 rpm in a tissue culture incubator. After achieving 80% confluence, cultures were transferred to 10% HS and placed back on the shaker. At the indicated times, each cell type was harvested separately for CK assays and protein determination.

**Transfection and Isolation of Dexamethasone-responsive Cell Lines**

Cultures of C2 cells in 10-cm culture dishes containing ~5 × 10^6 cells were transfected by calcium phosphate precipitation (19) with pSV2neo, which confers resistance to G418 as a selectable marker (31), and with the oncogenic form of N-ras containing a substitution of lysine for glutamine at amino acid 61 (4). The N-ras gene was under transcriptional control of the MMTV-LTR, conferring glucocorticoid inducibility to the gene. The details of this plasmid were described previously (35). At 16–24 h after transfection, cells were transferred from 1 10-cm dish to two 15-cm dishes and at 48 h after transfection 400 μg/ml of G418 (Gibco, Grand Island, NY) was added to the cultures in growth media. Individual colonies were isolated after 14 d and passed into stable cell lines. Clonal cell lines were exposed to a series of concentrations of dexamethasone from 10 nM to 2 μM and CK activity and the extent of fusion were determined. Cell lines that showed a dose-dependent inhibition or retardation of differentiation were selected and tested further.

**Assays for CK Activity and ACh receptor**

Creatine kinase (CK) was assayed as described previously (38). Acetylcholine receptor (AChR) were assayed by the specific binding of [125I]to-bungarotoxin to cell monolayers (39).
Assay for Expression of p21 ras Proteins by Western Transfer

A mouse anti-ras p21 monoclonal antibody, 259, (Cetus Corp., Emeryville, CA) was used for quantitation of p21 ras protein (15). Cell lysis and protein extraction were carried out according to manufacturer's instructions and protein transfer was performed as described previously (38). Equivalent quantities of protein from cells under each condition were applied to the lanes. After incubation of nitrocellulose blots with the primary anti-ras p21 antibody, an 125I-labeled rabbit anti-mouse secondary antibody was added. After subsequent washing, blots were exposed to Kodak X-AR5 film using intensifying screens. Relative levels of p21 ras were quantitated by densitometry of autoradiographs.

RNA Isolation and Northern Blot Hybridization

RNA was prepared from cell cytoplasm by magnesium precipitation of polyribosomes (44). The relative abundance of individual mRNAs was determined by Northern blot hybridization followed by densitometry, as described previously (52). Equivalent quantities of RNA from cells under each condition were applied to the lanes. N-ras mRNA was measured using a 665 base-pair Sal I/Eco RI fragment from a human N-ras cDNA containing exons II, III and IV (57, 58). DNA probes were labeled with 32P by the method of Feinberg and Vogelstein (10).

Results

N-ras Reversibly Inhibits Myoblast Fusion

When maintained at subconfluent densities in DME with 20% FCS, C2 myoblasts proliferate and exhibit none of the biochemical or morphological manifestations of the myogenic phenotype (25, 40, 41, 60). The onset of differentiation, after transfer of cultures at 80% confluence to medium with 10% HS (fusion-promoting medium), is marked by the appearance of multinucleate myotubes and the accumulation of muscle-specific gene products such as MCK and the ACh receptor.

Constitutive expression of transforming ras genes completely inhibits fusion and induction of muscle-specific gene products in C2 cells (41). To extend our understanding of the mechanisms involved in ras-dependent inhibition of myogenesis, C2 cells were transfected with a human N-ras gene, activated by a missense mutation at position 61 (4). Transcription of N-ras was controlled by the MMTV-LTR, rendering the gene inducible by dexamethasone. ras genes were co-transfected with the selectable marker pSV2neo, which confers resistance to the neomycin analog, G418. Colonies arising from the transfection were screened initially by adding fusion-promoting medium containing 2 µM dexamethasone. Of a total of 26 independent colonies examined, 10 failed to form myotubes when exposed to dexamethasone under fusion-promoting conditions. These dexamethasone-responsive colonies were isolated and passaged into stable cell lines. Subsequent analysis revealed that seven of the cell lines fused normally in the absence of dexamethasone but remained as mononucleate cells in the presence of dexamethasone. The remaining three of the clones were either partially or completely differentiation-defective.

In our previous studies using constitutively expressed ras oncogenes, we found that the effects of ras were dominant and that virtually all clones bearing mutant ras genes were unable to differentiate (41). Therefore, in the present study, we limited our analysis to clones that showed dose-dependent inhibition of morphological differentiation in response to dexamethasone. Clones that differentiated in the presence of dexamethasone were considered to lack functional steroid-inducible ras genes, whereas clones that never differentiated were assumed to express the transacted ras oncogene constitutively or to represent clones that originated from differentiation-defective cells in the original population.

Fig. 1 shows the morphology of a representative clonal cell line, designated CO25, bearing the steroid-inducible N-ras oncogene, under a series of culture conditions. In growth medium, CO25 cells were morphologically indistinguishable from nontransfected C2 myoblasts (data not shown). Transfer of cultures at 80% confluence to fusion-promoting medium led to formation of myotubes with kinetics identical to normal C2 cells (Fig. 1A). In contrast, addition of 100–2,000 nM dexamethasone to fusion-promoting medium resulted in a complete block in the ability of CO25 myoblasts to form myotubes (Fig. 1B). The inhibitory effects of dexamethasone were attenuated by subconfluent densities in DME with 20% FCS, C2 myoblasts proliferate and exhibit none of the biochemical or morphological manifestations of the myogenic phenotype (25, 40, 41, 60). The onset of differentiation, after transfer of cultures at 80% confluence to medium with 10% HS (fusion-promoting medium), is marked by the appearance of multinucleate myotubes and the accumulation of muscle-specific gene products such as MCK and the ACh receptor.

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Figure 1. Effects of dexamethasone on morphology of myoblasts bearing steroid-inducible N-ras oncogenes. The CO25 cell line, bearing the steroid-inducible N-ras oncogene, was cultured in DME with 20% FCS. After achieving 80% confluence, cultures were transferred to fusion-promoting medium for 5 d (A). A separate set of cultures was treated identically but was exposed to 500 nM dexamethasone 48 h before transfer to fusion-promoting medium containing dexamethasone (B). After 3 d in fusion-promoting medium containing dexamethasone, cultures shown in Panel B were transferred to fresh medium without dexamethasone for 3 d (C).
were subject to regulation by dexamethasone, undifferentiated cultures in growth medium were exposed to 500 nM dexamethasone for 48 h before transfer to fusion-promoting medium without dexamethasone on morphological differentiation were reversible, indicating that dexamethasone inhibited rather than delayed the morphological alterations associated with terminal differentiation. In the presence of dexamethasone at concentrations lower than 100 nm, intermediate levels of myoblast fusion were observed (data not shown).

To determine whether the inhibitory effects of dexamethasone on morphological differentiation were reversible, fusion-blocked CO25 myoblasts, maintained in the presence of fusion-promoting medium with dexamethasone for 3 d, were transferred to fusion-promoting medium without dexamethasone. As shown in Fig. 1 C, these cultures formed large branching myotubes within 3 d after removal of dexamethasone. Thus, the inhibitory effects of dexamethasone on morphological differentiation of myoblasts bearing steroid-inducible ras oncogenes were fully reversible and required the continual presence of the steroid in the culture medium.

**Transforming N-ras Oncogenes Inhibit Muscle-specific Gene Expression**

To determine whether muscle-specific genes in CO25 cells were subject to regulation by dexamethasone, undifferentiated cultures in growth medium were exposed to 500 nM dexamethasone for 48 h before transfer to fusion-promoting medium, or were transferred directly to fusion-promoting medium containing the steroid. In the absence of dexamethasone, the muscle-specific gene products, CK and ACh receptor, were induced in a similar manner in normal C2 cells and in CO25 cells (Fig. 2). In the presence of dexamethasone, however, induction of CK and ACh receptors was inhibited dramatically in CO25 cells. Suppression of differentiation by dexamethasone occurred rapidly as demonstrated by the nearly complete inhibition of expression of muscle-specific gene products in cultures exposed to fusion-promoting medium with dexamethasone without pretreatment with the steroid (Fig. 2, C and D). Induction of CK activity and ACh receptors in C2 myoblasts was not inhibited by dexamethasone (Fig. 2, A and B). Together, these results demonstrate that the morphological and biochemical events associated with myogenesis become sensitive to inhibition by dexamethasone after transfection of myoblasts with a steroid-inducible ras oncogene.

**Time Course for Induction of N-ras Protein and mRNA by Dexamethasone**

The ability of dexamethasone to inhibit differentiation of CO25 myoblasts with no apparent lag (Fig. 2) suggested that the N-ras protein accumulated relatively rapidly in response to steroid induction. To determine the kinetics for ras induction, CO25 myoblasts were exposed to 500 nM dexamethasone for a series of times, cell extracts were prepared, and levels of p21 ras protein were quantitated by Western blot analysis using the anti-ras monoclonal antibody, 259 (15). In the absence of dexamethasone, a 21-kD polypeptide that comigrated with purified ras protein, was evident in CO25 cells (Fig. 3 A) and in C2 myoblasts (data not shown). Because the antibody used for these studies recognizes the endogenous proto-oncogenic forms of H-ras and N-ras from mouse, we conclude that this band represents one or both of these normal ras proteins. We do not know the identity of the polypeptide of higher molecular weight that is sometimes detected by the anti-ras antibody. After addition of dexamethasone to CO25 cells, the p21 polypeptide began accumulating ~3 to 5 h after exposure of cells to the steroid and continued to increase over a period of 48 to 60 h. The maximum steady state level achieved by exogenous p21 ras was four- to fivefold higher than the level of the endogenous ras proteins (Fig. 3, A and B).

The kinetics for induction of N-ras mRNA also were determined by Northern blot hybridization using a 32P-labeled human N-ras cDNA. The hybridization probe used for this experiment hybridizes only to human N-ras transcripts and therefore permits accurate measurement of exogenous N-ras mRNA expression without complications from the endogenous proto-oncogenic mRNA species. As shown in Fig. 3 C this probe hybridized to an mRNA species of 2.2 kb, corresponding to exogenous N-ras mRNA (21, 35). The kinetics of induction of N-ras mRNA were similar to those of p21 N-ras protein. The pattern of expression of N-ras mRNA in response to dexamethasone was similar in myoblasts growing in 20% FCS (Fig. 3 C) and in differentiating cultures after 2 d in 10% HS (data not shown).

It is of interest to note that steroid induction of N-ras via the MMTV-LTR occurred with delayed kinetics in C2 cells compared with previous reports in 3T3 cells (26, 43). We also observed little or no feed-back inhibition of ras expression as was observed in 3T3 cells transfected with MMTV-
LTR-driven H-ras genes. The molecular basis for the contrasting pattern of expression of N-ras in C2 myoblasts is unclear, but may reflect a lower level of glucocorticoid receptors in these cells, although this has not yet been tested.

Dose-dependent Induction of N-ras and Inhibition of Differentiation by Dexamethasone

It was of interest to correlate directly the levels of N-ras expression with the extent of differentiation and thereby determine the minimum level of ras required to completely extinguish the differentiated phenotype. The dose dependence for inhibition of differentiation of CO25 cells by dexamethasone is shown in Fig. 4 A. Partial inhibition of CK expression was observed at concentrations of dexamethasone as low as 5 nM. The ID50 for dexamethasone was ~7 nM, with maximum inhibition occurring at 100 nM. These results indicate that ras does not inhibit myogenesis through an absolute all-or-none event. Instead, it appears that the effects of ras occur in a graded manner through a concentration-dependent mechanism.

The dose-dependence on dexamethasone of N-ras mRNA induction was also analyzed in CO25 cultures exposed to varying concentrations of the steroid for 72 h. As shown in Fig. 4 B, N-ras mRNA was detectable in the presence of dexamethasone at concentrations as low as 5 nM. Maximal levels of N-ras mRNA expression occurred at 25 to 100 nM dexamethasone. Comparison of the dependence on dexamethasone of N-ras mRNA accumulation with the extent of inhibition of differentiation, showed an inverse relationship (Fig. 4 C); the EDso for both processes was 7 nM with maximal responses occurring between 25 and 100 nM.

Reversibility of Ras-dependent Inhibition of Differentiation

To determine whether the inhibitory effects of ras on muscle-specific gene expression were reversible, CO25 cells were maintained in fusion-promoting medium containing 500 nM dexamethasone for 3 d and were subsequently transferred to fresh fusion-promoting medium lacking the steroid. As shown in Fig. 5, removal of dexamethasone from differentiation-inhibited CO25 cells led to appearance of CK and ACh receptors with kinetics indistinguishable from those of the initial induction of these muscle gene products in C2 cells or in non-dexamethasone-treated CO25 cells. These results indicate that the inhibitory effects of ras on muscle-specific gene expression are readily reversible and that prolonged expression of ras in myoblasts neither accelerates nor delays subsequent induction of muscle-specific gene products after release of myoblasts from ras-dependent inhibition.

Terminal Differentiation After Release from ras-dependent Repression Does Not Require a Round of DNA Synthesis

It was of interest to determine whether CO25 myoblasts, released from ras-dependent inhibition of differentiation by removal of dexamethasone, required a round of cell division to be reprogrammed before induction of the differentiated phenotype. To address this question, we tested whether inhibition of DNA synthesis interfered with differentiation of CO25 cells after transfer from fusion-promoting medium containing dexamethasone to fresh medium lacking the ste-
roid. In the presence of 5 μg/ml aphidicolin, which inhibits DNA synthesis, fusion (data not shown) and induction of CK activity occurred normally after removal of dexamethasone from differentiation-inhibited cultures (Fig. 6). Similar results were obtained in the presence of 0.1 mM cytosine arabinoside, which eliminates replicating cells (data not shown). It can be concluded, therefore, that a round of DNA synthesis is not required for myoblasts to overcome the inhibitory effects of ras on differentiation.

The N-ras Oncogene Does Not Abrogate the Requirement for Growth Factors to Divide

Differentiation of skeletal myoblasts depends absolutely upon cessation of cell division. Because transforming ras genes have been reported to release some cell types from their dependence on exogenous growth factors to divide, it was important to determine whether the failure of CO25 cells to differentiate in the presence of dexamethasone might be attributable to continued proliferation of these cells in fusion-promoting medium. As shown in Fig. 7, neither the rate of cell division in 20% FCS nor the saturation density achieved by CO25 cells were affected appreciably by dexamethasone. Moreover, dexamethasone-treated CO25 cells withdrew from the cell cycle at subconfluent densities in medium with 0.5% FCS in a manner identical to C2 myoblasts. These experiments were performed in medium containing 0.5% FCS rather than 10% HS because HS contains a low level of mitogenic activity that supports a slow rate of growth of C2 myoblasts at low densities. To determine whether CO25 cells were able to stop dividing completely in the presence of dexamethasone it was necessary to analyze their growth rate under conditions in which there was no proliferation in control cultures. The inhibitory effects of
Figure 5. Reversibility of the differentiation-defective phenotype of myoblasts bearing steroid-inducible N-ras oncogenes. The CO25 cell line, bearing the steroid-inducible N-ras oncogene, was cultured in DME with 20% FCS. After achieving 80% confluency (day 0), cultures were transferred to fusion-promoting medium in the presence (o) or absence (●) of 500 nM dexamethasone (DEX). Half of the cultures maintained in dexamethasone were transferred to fresh fusion-promoting medium without dexamethasone on day 3 (▲). CK activity and ACh receptors were assayed on consecutive days.

Dexamethasone on differentiation of CO25 cells were also observed in the presence of 0.5% FCS (data not shown). We conclude that dexamethasone-dependent inhibition of differentiation of CO25 cells occurs through a mechanism independent of continuous cell proliferation. These results do not rule out the possibility, however, that ras may confer subtle alterations in sensitivity to specific growth factors that would not be detectable by the assays employed here.

Ras Does Not Inhibit Myogenic Differentiation through An Autocrine Mechanism

The ability of ras oncogene proteins to inhibit myogenic differentiation could be due to direct activation by ras of an intracellular pathway that interferes with muscle-specific gene expression. Alternatively, ras could prevent myogenesis indirectly through an autocrine mechanism involving release of an inhibitory growth factor. The latter possibility is particularly relevant, in light of reports that some ras-transformed cell types secrete transforming growth factors (1, 54), which are known to inhibit myogenesis (14, 33, 40). To distinguish between these possibilities, normal C2 cells and CO25 cells were cultured on opposite sides of a barrier that allowed free exchange of medium between the two cell types. Cultures were maintained in growth medium containing 2 μM dexamethasone for 48 h and were subsequently transferred to fusion-promoting medium with the steroid for 3 d. Cells were then harvested separately from each side of the permeable barrier and CK activity was assayed. As shown in Fig. 8, differentiation of C2 cells was not affected significantly by the presence of medium conditioned by dexamethasone-treated CO25 cells. These results argue against the involvement of autocrine factors in ras-dependent inhibition of differentiation and suggest that ras activates an intracellular cascade that interferes directly with the differentiation program.

Figure 6. Effects of inhibitors of DNA synthesis on induction of CK activity following removal of dexamethasone from myoblasts bearing steroid-inducible N-ras oncogene. The CO25 cell line, bearing the steroid-inducible N-ras oncogene, was cultured in DME with 20% FCS. After achieving 80% confluency, cultures were transferred to fusion-promoting medium in the presence (o) or absence (●) of 500 nM dexamethasone (DEX). After 3 d, dexamethasone-treated cultures were transferred to fresh fusion-promoting medium lacking the steroid, but containing 5 mM aphidicolin (▲) or no addition (●). CK activity was determined on consecutive days.

Induction of N-ras in Terminally Differentiated Myotubes Does Not Extinguish Muscle-specific Gene Expression

The ability to modulate N-ras expression in CO25 cells with dexamethasone made it possible to investigate whether in-
production of N-ras in terminally differentiated myotubes was sufficient to extinguish expression of muscle-specific genes. Preliminary experiments indicated that myotube cultures that contained a significant percentage of nuclei in unfused myocytes showed a decline in expression of CK activity and mRNA in response to dexamethasone. We believe this inhibitory response may reflect the down-regulation of muscle-specific gene products in partially differentiated mononucleate cells, since fusion is not a prerequisite for muscle-specific gene expression in C2 cells (Hu, J. S., and E. N. Olson, unpublished results). Our primary purpose was to examine whether muscle-specific genes in terminally differentiated myotubes were susceptible to ras-dependent repression. Therefore, to obtain cultures of pure myotubes, CO25 cells were cultured in fusion-promoting medium for 2 d, at which time large, branching myotubes were clearly visible. Nonfused myocytes were then eliminated from the cultures by stimulation with growth medium containing 20% FCS and 0.1 mM cytosine arabinoside for 2 d. Pure cultures of terminally differentiated myotubes were exposed subsequently to 1 μM dexamethasone and CK activity was assayed at 24-h intervals after addition of the steroid. Under these conditions, CK activity (Fig. 9) and MCK mRNA (data not shown) continued to increase after addition of dexamethasone to cultures of CO25 myotubes. The morphology of CO25 myotubes also remained unchanged in the presence of dexamethasone.

The failure of ras to suppress muscle-specific gene expression in myotubes cannot be attributed to a loss of responsiveness of the transfected ras gene to steroids because N-ras mRNA was induced in myotubes in response to dexamethasone (Fig. 10). We did observe, however, that 1,000 nM dexamethasone was required to induce ras expression in myotubes to levels comparable to that induced in myoblasts by 100 nM dexamethasone (data not shown). Together these results demonstrate muscle-specific genes are susceptible to ras-dependent repression during the early stages of the differentiation process. However, in pure cultures of terminally differentiated myotubes muscle-specific genes appear to become refractory to the inhibitory signals generated by transforming ras proteins.

Discussion

The transition of certain types of myoblasts to terminally differentiated myotubes has been shown previously to be inhibited by TGFβ and FGF through a mechanism independent of cell proliferation (6, 14, 28, 29, 33, 40, 52). Inhibition of fusion and muscle-specific gene expression requires the continual presence of these growth factors in the extracellular milieu and is rapidly reversible after their removal. These results are consistent with the notion that inhibition of myogenesis by TGFβ and FGF requires continuous occupancy of their respective cell surface receptors and involves an intracellular signal (or signals) that is extremely short-lived.

In an effort to identify the intracellular signalling pathway(s) used by TGFβ and FGF to regulate myogenesis, we have introduced a series of mutationally activated ras oncogenes into myoblasts and have investigated whether the proteins encoded by these oncogenes might confer a phenotype on myoblasts similar to that elicited by TGFβ and FGF. ras oncogenes were selected for these studies because ras proteins are localized to the plasma membrane where they are believed to function at an early step in intracellular growth factor cascades by transducing signals between specific cell surface growth factor receptors and intracellular enzyme effectors (2, 11, 27, 30, 35, 37, 55, 59). Recently, we reported that transfection of C2 cells with mutationally activated N- or H-ras oncogenes, under constitutive transcriptional control, completely inhibited the molecular events associated with myogenic differentiation (41). Similar inhibition of differentiation by ras was observed in the BC3H1 muscle cell line (5, 46, Klein, D. J., and A. Connally, personal communication).

In the present study, we have extended these previous observations by constructing myoblast cell lines in which the entire developmental program is subject to negative control
Figure 10. Effects of dexamethasone on expression of N-ras mRNA in terminally differentiated myotubes. CO25 cells were allowed to achieve 80% confluency and were transferred to medium with 10% FCS and 0.1 mM cytosine arabinoside to eliminate unfused cells. Dexamethasone (1 μM) was also added to the cultures at the time of addition of cytosine arabinoside. At the indicated times, RNA was isolated and steady state levels of N-ras mRNA were determined by Northern blot analysis. Levels of N-ras mRNA in myoblasts exposed to 1 μM dexamethasone and in myotubes in the absence of the steroid are also shown.

by dexamethasone. These myoblast cell lines, bearing steroid-inducible N-ras oncogenes, permit cell cycle withdrawal to be reversibly dissociated from muscle-specific gene induction and allow fine-tuning of the differentiated phenotype in a manner not previously possible. The precise control over the differentiation program afforded by these cell lines should facilitate detailed analysis of the mechanisms whereby growth factor signalling cascades impinge on muscle-specific genes.

The results of the present study extend our understanding of the mechanism for ras-dependent regulation of myogenesis in at least four important respects. First, suppression of differentiation of myoblasts bearing steroid-inducible ras oncogenes was demonstrated to be rapidly reversible after withdrawal of dexamethasone. This observation indicates that the ras-dependent phenotype requires the continual presence of ras to be maintained and implies that interference with the differentiation program by ras proteins requires the persistent transduction of highly transient intracellular signals. Second, the degree of differentiation of myoblasts bearing steroid-inducible ras genes was a titratable function of N-ras expression with partial inhibition of fusion and induction of muscle-specific genes occurring at low levels of ras. These results indicate the ras does not exert an all-or-none effect on the differentiation program, but rather, elicits a graded response proportional to the level of ras expression. Third, after release of myoblasts from ras-dependent inhibition, differentiation proceeded with normal kinetics, suggesting that ras arrests myoblasts at an early, rather than late step in the pathway toward differentiation. Induction of differentiation after release from ras-dependent inhibition also was shown to occur in the absence of an additional round of DNA synthesis, indicating that myoblasts do not need to be reprogrammed during a quantal cell cycle to overcome the differentiation-defective phenotype conferred by ras. Finally, induction of N-ras failed to down-regulate muscle-specific genes in terminally differentiated myotubes. In each of these respects, the effects of oncogenic ras proteins on myogenesis resemble the effects of TGFβ and FGF.

Several transformed cell types have been reported to secrete transforming growth factors (1, 54). Therefore, to test the possibility that ras-dependent inhibition of myogenesis might be mediated by an autocrine growth factor similar, or identical, to TGFβ or FGF, the ability of ras-transfected myoblasts to transfer their phenotype to normal myoblasts through culture medium was examined. No evidence for autocrine interactions between ras-transformed and normal myoblasts was detectable. Thus, we conclude that oncogenic ras proteins directly transduce an intracellular signal that operates in an antagonistic manner to the normal developmental signals that initiate myogenesis. It should be emphasized that ras proteins do not function directly as muscle-specific gene repressors. Instead, these proteins most likely activate an intracellular pathway that culminates in repression of the differentiation program.

What type of mechanisms might be responsible for ras-dependent inhibition of myogenic differentiation and how many intermediate steps may be involved? ras proteins are localized to the plasma membrane where they are postulated to function at an early step in the pathway for transduction of intracellular growth factor signals. Mutationally activated ras proteins have been reported to substitute for exogenous growth factors and to persistently activate intracellular signalling pathways that may normally be modulated by proto-oncogenic ras proteins (30). In light of the similarities between the effects of TGFβ, FGF and ras on myogenesis, it is tempting to speculate that signalling pathways similar or identical to those activated by TGFβ and FGF might be amplified in myoblasts bearing oncogenic ras genes. In this regard, several of the early events associated with growth factor receptor occupancy have been shown to be activated by oncogenic ras proteins. For example, microinjection of fibroblasts with oncogenic ras proteins or transfection with ras genes leads to increased activity of phospholipases A2 and C, which modulate arachidonate release and phosphatidylinositol turnover, respectively (2, 13, 47, 59). ras oncogene proteins also have been reported to lead to an increase in intracellular pH, due to stimulation of the amiloride-sensitive Na+/H+ exchange (20). Finally, ras transformed cells have been demonstrated to exhibit increased rates of glycolysis and hexose transport which may contribute to a generalized alteration in cellular metabolism (48). The potential involvement of one or more of these biochemical events in ras-dependent repression of myogenesis is currently under investigation.
Terminal differentiation of mouse MM14 myoblasts has been shown by Hauschka and coworkers to be preceded by down-regulation of cell surface EGF and FGF receptors (31, 42). A similar pattern of regulation has been observed for TGFβ receptors during fusion of C2 and L6A1 myoblasts (8). This loss of cell surface growth factor receptors has been postulated as a mechanism to account for the inability of growth factors to suppress muscle-specific gene expression in myotubes. Because ras proteins are believed to function at a post-receptor step in growth factor cascades, it was possible to use CO25 cells to investigate whether muscle-specific genes in myotubes remained susceptible to repression by intracellular growth signals. The results reported here, which show that pure myotubes are insensitive to signals generated by ras proteins, suggest that irreversible activation of muscle-specific genes in myotubes may not result exclusively from down-regulation of growth factor receptors. The nature of the molecular events that place muscle-specific gene expression in myotubes beyond the control of ras remain to be established.

The consequences of expression of oncoproteins encoding proteins presumed to function at early steps in intracellular growth factor cascades have been examined previously in cultures of primary myoblasts (9, 23, 24, 36). These studies demonstrated that v-src and v-erbB, which encode tyrosine protein kinases, prevent the molecular events associated with myogenesis. Like the effects of ras reported here, these oncogene products prevented differentiation through a mechanism independent of cell proliferation. It remains to be determined whether tyrosine kinases and mutationally activated ras proteins function through the same, or through parallel pathways to elicit their effects on the differentiation program.

Myoblast cell lines, modified by incorporation of exogenous oncogenes, offer the opportunity to dissect the mechanisms whereby intracellular signals generated at the cell surface culminate in alterations of gene expression. In the future, it will be particularly interesting to determine whether ras functions in the same intracellular pathway activated by TGFβ and FGF or whether these regulators of myogenesis utilize separate pathways that converge at a downstream step that governs the differentiation program.

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