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Signaling through Mitogen-Activated Protein Kinase and Rac/Rho Does Not Duplicate the Effects of Activated Ras on Skeletal Myogenesis

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The ability of basic helix-loop-helix muscle regulatory factors (MRFs), such as MyoD, to convert nonmuscle cells to a myogenic lineage is regulated by numerous growth factor and oncoprotein signaling pathways. Previous studies have shown that H-Ras 12V inhibits differentiation to a skeletal muscle lineage by disrupting MRF function via a mechanism that is independent of the dimerization, DNA binding, and inherent transcriptional activation properties of the proteins. To investigate the intracellular signaling pathway(s) that mediates the inhibition of MRF-induced myogenesis by oncogenic Ras, we tested two transformation-defective H-Ras 12V effector domain variants for their ability to alter terminal differentiation. H-Ras 12V,35S retains the ability to activate the Raf/MEK/mitogen-activated protein (MAP) kinase cascade, whereas H-Ras 12V,40C is unable to interact directly with Raf-1 yet still influences other signaling intermediates, including Rac and Rho. Expression of each H-Ras 12V variant in C3H10T1/2 cells abrogates MyoD-induced activation of the complete myogenic program, suggesting that MAP kinase-dependent and -independent Ras signaling pathways individually block myogenesis in this model system. However, additional studies with constitutively activated Rac1 and RhoA proteins revealed no negative effects on MyoD-induced myogenesis. Similarly, treatment of Ras-inhibited myoblasts with the MEK1 inhibitor PD98059 revealed that elevated MAP kinase activity is not a significant contributor to the H-Ras 12V effect. These data suggest that an additional Ras pathway, distinct from the well-characterized MAP kinase and Rac/Rho pathways known to be important for the transforming function of activated Ras, is primarily responsible for the inhibition of myogenesis by H-Ras 12V.

The ability to achieve the complete differentiation of skeletal muscle precursor cells in culture has provided a model system in which to characterize the extracellular and intracellular molecules that regulate the proper progression of the myogenic program. The conversion of noncommitted cells to a myogenic fate is determined, in part, by the transcriptional activities of the four basic helix-loop-helix muscle regulatory factors (MRFs) MyoD, Myf-5, myogenin, and MRF4. The MRFs are expressed early in development to establish the myogenic lineage, and they function later in development, along with ubiquitously expressed basic helix-loop-helix E-protein binding partners, to transcriptionally activate muscle-specific genes (21, 35). The activities of the MRFs, in turn, are regulated by environmental cues, such as exposure of cells to serum mitogens or to the purified growth factors fibroblast growth factor 2 (FGF-2) and transforming growth factor β1 (1, 23, 26). Implicit in this observation is the critical role of intracellular signaling pathways, which serve to translate the influence of these extracellular stimuli into intracellular enzymatic activities that, in the case of serum mitogens, FGF-2, and transforming growth factor β1, negatively impact MRF function and inhibit differentiation to a skeletal muscle lineage.

The plasma membrane-localized Ras family of GTPases occupies a pivotal position in intracellular signal transduction by relaying signals generated from transmembrane receptor protein tyrosine kinases to downstream signaling molecules, including the Raf-1 kinase (12, 29), PI3 kinase (8, 34, 36), Ral-GDS (40, 46), and the Rac and Rho GTPases (8, 17, 32, 33). The mutational activation of Ras, in which constitutive Ras signaling causes dramatic changes both in cellular gene transcription (8, 25, 47) and in cellular architecture (16), confers on cells a distinct growth advantage and is among the most prevalent genetic alterations contributing to human cancer (4). Not surprisingly, numerous studies have demonstrated the ability of an oncogenic Ras protein to inhibit skeletal myogenesis (11, 19, 20, 22, 30), presumably by mimicking the signaling events generated following the exposure of myoblast populations to serum growth factors. Interestingly, while it is well documented that all of the aforementioned molecular inhibitors of myogenesis target MRF activity (1, 5, 13, 19, 24, 28, 41, 42), the mechanism of this inhibition varies. In the case of FGF-2 signaling, the phosphorylation of a conserved threonine residue in myogenin prevents DNA binding (24). On the other hand, FGF-2 does not induce hyperphosphorylation of the corresponding threonine residue in MRF4, and the myogenic activity of MRF4 or MyoD proteins lacking this threonine is still inhibited by FGF-2 (13). Additionally, in stable C3H10T1/2-Ras cells transiently transfected with MRF4 or MyoD, neither the formation of MRF-E12 heterodimers on target DNA nor the intrinsic ability of these complexes to transcriptionally activate a simple, muscle-specific reporter gene is inhibited (19). These findings suggest that a signaling pathway activated in response to oncogenic Ras expression disrupts additional uncharacterized MRF-protein interactions that have been implicated by several MRF-targeted mutagenesis studies (19). The inability of previous studies to identify the molecules blocking MRF activity in response to growth factors or onco-

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genic Ras underscores the need for further investigation of the cytoplasmic signaling pathways utilized by various negative regulators of myogenesis.

To further define the downstream components of Ras signal transduction that negatively impact the differentiation of skeletal muscle cells, we have investigated the role of intracellular signaling pathways known to be essential for complete cellular transformation by activated Ras (16). We used two transformation-defective Ras effector domain variants (one is blocked in signaling through Raf/MEK/mitogen-activated protein [MAP] kinase, and the other retains the ability to interact with Raf-1 and to activate the MAP kinase pathway [45]) and found that both are competent to inhibit the biochemical and morphological differentiation of C3H10T1/2 fibroblasts expressing MyoD. Our initial interpretation of these results was that myogenesis in this system is blocked by the individual activation of the Ras signaling pathways distinguished by these two effector domain variants. However, additional experiments demonstrated that this was not the case. Despite documented roles in Ras-mediated cellular transformation events (6, 17, 32, 33), expression of constitutively active Rac1, RhoA, or MEK1 proteins in C3H10T1/2 cells does not restrict the ability of MyoD to induce myogenic differentiation. Furthermore, blocking the Ras-induced activation of MAP kinase with the MEK1 inhibitor PD98059 does not reverse the nonmyogenic phenotype of Ras-expressing cells. Based on these additional findings, we concluded that the well-characterized signaling events which are essential for complete cellular transformation by activated Ras do not play a major role in the Ras-mediated inhibition of skeletal myogenesis and that a third, yet-to-be-defined complement of signaling molecules mediates the Ras-induced block of MRF activity in this model system.

MATERIALS AND METHODS

Expression constructs. TnI-Luc, a luciferase reporter gene controlled by the muscle-specific quail troponin I enhancer (15), and SRF2-Luc, a luciferase reporter gene regulated by a single serum response factor binding site from the c-*fos* promoter (14), have been described previously. pG5T-Luc ($Gal4_5$-Luc), a luciferase reporter gene containing five tandem Gal4 DNA binding sites, was obtained from J. D. Galla. The expression plasmid pEMc11S (pEM-MyoD) contains mouse MyoD cDNA (19), while Gal4-Elk1 is a fusion protein consisting of the Gal4 DNA binding domain and the transcription activation domain of Elk1 (27). Human H-*ras* cDNAs altered by site-directed mutagenesis were constructed as described previously (45) and were expressed as hemagglutinin (HA)-tagged fusion proteins using the cytomegalovirus (CMV) expression cassette in pDCR, a vector which also expresses a bacterial neomycin resistance gene. Human Raf-1 cDNA and its derivative Raf-1 CAAX were expressed as Myc epitope-tagged fusion proteins in the expression vector pMT2. pZIPSV(X)1 vectors, containing cDNAs encoding the constitutively active human Rac1 (Rac1 115I) and RhoA (RhoA 63L) proteins, have been described previously (17). The human MEK1 cDNAs, pBABE MEK E217/E221 and pBABE MEK A221 (6), encoding a constitutively active MEK1 protein and an interfering, dominant negative MEK1 protein, respectively, were obtained from Christopher Marshall.

Cell cultures and transfections. C3H10T1/2 mouse fibroblasts were maintained in growth medium consisting of basal modified Eagle medium (GIBCO) supplemented with 10% fetal bovine serum (Bio-Whittaker), penicillin (100 U/ml), and streptomycin (100 μg/ml). To induce myogenesis, transfected cultures were exposed to differentiation medium (DM) composed of Dulbecco's low-glucose modified Eagle medium supplemented with 2% horse serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) for 48 h. Stable C3H10T1/2 transfectants were maintained in growth medium containing 400 μg of Geneticin (G418; GIBCO) per ml.

Transient cell transfections were carried out by calcium phosphate-DNA precipitation as described previously (15, 19) with the amounts of plasmid DNA indicated in the legends to Fig. 1 through 7. DNA precipitates were added for 4 h, after which the cells were osmotically shocked with 20% glycerol in basal modified Eagle medium for 2 min, maintained in growth medium for 18 h, and then treated with DM for 48 h. Where indicated, a 20 mM stock of PD98059 (New England Biolabs) in dimethyl sulfoxide was added to the DM to achieve a final concentration of 50 μM. For gene expression studies, cells were harvested in lysis buffer (25 mM Tris [pH 7.8], 4 mM EDTA, 1% Triton X-100, 10% glycerol). Following normalization for total protein content (Bio-Rad protein assay), extracts were analyzed with the luciferase assay kit (Promega). A minimum of three independent transfections were performed for each experimental group. Parallel cultures were processed for immunocytochemistry and for Western blot analysis as described below.

Stable transfection of C3H10T1/2 cells was performed as described previously (7) with 50 ng of the pDCR H-Ras constructs indicated below or 50 ng of pKOneo (39) and 150 ng of the pMT Raf-1 constructs. Twenty-four hours following transfection, the cultures were split into six 100-mm culture dishes and maintained in growth medium plus 400 μg of G418 per ml. After approximately 12 days, the $G418^r$ colonies from each experimental group were combined and maintained as a stably transfected pooled population.

Immunocytochemistry. C3H10T1/2 cells transfected and treated with DM were fixed in a solution of 70% ethanol-formalin-acetic acid (20:2:1), permeabilized with 0.1% Nonidet P-40 in 10 mM Tris (pH 8.0)–150 mM NaCl (TBS), and blocked by incubation with 2% horse serum in phosphate-buffered saline. The cells then were incubated with MF-20, a monoclonal antibody specific for the myosin heavy-chain (MHC) protein (3), and visualized with a secondary antibody and Vectastain kit reagents (Vector Laboratories). The number of myosin-positive cells was averaged following the examination of 10 randomly chosen microscope fields and, in all instances, is expressed as a percentage of the number of myofiber-forming C3H10T1/2 cells transfected with pEM-MyoD alone.

Western blot analysis. Transfected C3H10T1/2 cells were harvested in 4× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (200 mM Tris [pH 6.8], 400 mM dithiothreitol, 8% SDS, 0.4% bromophenol blue, and 40% glycerol) and normalized for protein content. Equal amounts of protein were separated by SDS–12% PAGE and transferred to nitrocellulose filter paper as described previously (19). Nonspecific binding sites were blocked by incubation with 5% nonfat dry milk in TBST (10 mM Tris [pH 8.0], 150 mM NaCl, 0.1% Tween 20) followed by incubation in 5% nonfat dry milk in TBST containing a 1:300 dilution of anti-MyoD (C-20; Santa Cruz), a 1:5,000 dilution of anti-Myc (9E10; gift of Robert Geahlen), a 1:1,000 dilution of anti-HA (Boehringer Mannheim), a 1:100 dilution of anti-Rac 1 (C-11; Santa Cruz), a 1:100 dilution of anti-RhoA (26C4; Santa Cruz), or a 1:300 dilution of anti-MEK1 (C-18; Santa Cruz). Following several washes in TBST, reactions were detected by using a peroxidase-conjugated secondary antibody and enhanced chemiluminescence (ECL kit; Amersham).

In vitro MAP kinase assays. C3H10T1/2 cells transfected with the constructs indicated in the legends to Fig. 1 through 7 were maintained for 48 h in DM containing 50 μM PD98059 or an equal volume of dimethyl sulfoxide. Cells were harvested in homogenizing buffer (HB) (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 5 mM *p*-nitrophenylphosphate-di-Tris salt, 1 mM sodium orthovanadate, 10 mM tetrasodium pyrophosphate decahydrate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg each of aprotinin, leupeptin, and pepstatin per ml). The cells were sonicated, and each supernatant was normalized for protein concentration. Radioimmunoprecipitation assay (RIPA) detergent concentrate (10×) (HB containing 10% Triton X-100, 1% SDS, and 5% deoxycholate) was added to each extract to yield a final concentration of 1× RIPA detergent. MAP kinase was immunoprecipitated from the lysates by addition of 20 μl of protein A-agarose and 10 μl of rabbit anti-MAP kinase. The activity of the MAP kinase in the immunocomplex was determined by using myelin basic protein (MBP) as a substrate in a reaction buffer composed of 20 mM Tris (pH 7.5), 10 mM $MgCl_2$, 5.4 mM pNPP-di-Tris, and 2.5 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; Amersham). Reactions were terminated after 30 min at 30°C by addition of sample buffer. Proteins were resolved by SDS–12% PAGE and visualized by autoradiography.

RESULTS

Transformation-defective Ras proteins inhibit myogenesis. Previous studies have demonstrated that the constitutively active form of Ras (H-Ras 12V) inhibits both muscle-specific gene transcription and the formation of multinucleate myofibers (11, 19, 20, 22, 30). The downstream effectors of Ras signaling in cells include the components of at least two distinct pathways, the Raf/MEK/MAP kinase cascade (8, 29) and a pathway initiated by the activation of Rac and Rho, resulting in alterations in cellular architecture (16). Studies from a number of laboratories have shown that Ras-mediated cellular transformation relies on the synergy of Raf-1 and Rac/Rho signaling events (17, 32, 33). Site-directed mutagenesis of the Ras effector domain (45) has produced molecular reagents which are transformation defective or attenuated due to alterations in effector interactions. To determine the contribution of distinct Ras-stimulated signaling pathways to the inhibition of myogenesis by H-Ras 12V, C3H10T1/2 fibroblasts were transiently transfected with a muscle-specific luciferase reporter gene (TnI-Luc) and expression vectors encoding mouse MyoD (pEM-MyoD) and one of the following Ras proteins: H-Ras

12V (wild-type, activated Ras), H-Ras 12V,35S (an effector domain mutant which stimulates Raf-1), and H-Ras 12V,40C (an effector domain mutant which does not interact with Raf-1 yet induces membrane ruffling characteristic of Rac activation) (16, 45). Twenty-four hours following transfection, the cells were treated with DM and incubated for an additional 48 h prior to the preparation of cell lysates. As shown in Fig. 1A, C3H10T1/2 cells transfected with MyoD and the muscle-specific reporter gene generated significant levels of transcriptional activity, while H-Ras 12V inhibited TnI-Luc activity by over 90%. Interestingly, cells cotransfected with TnI-Luc, pEM-MyoD, and either H-Ras 12V,35S or H-Ras 12V,40C also failed to exhibit significant levels of muscle-specific gene transcription, implying that activation of either Ras downstream signaling pathway negatively impacts skeletal muscle differentiation. Indeed, the complete myogenic program in these cells is inhibited by all three Ras proteins as revealed by MHC immunostaining of parallel differentiation-induced cultures (Fig. 1B). To confirm that all constructs were expressed appropriately in these cells and that repression of myogenesis by individual Ras proteins was not due to an inhibition of MyoD protein accumulation, whole-cell lysates prepared from transfected C3H10T1/2 cells were analyzed by Western blotting with anti-HA and anti-MyoD antibodies. As predicted, each of the HA-tagged Ras proteins was expressed under differentiation conditions (Fig. 1C). Similarly, the level of MyoD was equivalent in the myogenic-competent (control) and myogenic-incompetent (H-Ras 12V-, H-Ras 12V,35S-, and H-Ras 12V,40C-transfected) cultures. Importantly, the block in myogenesis by the H-Ras 12V constructs was not due to a general inhibition of transcription in C3H10T1/2 cells, since H-Ras 12V has no effect on expression of several control reporter genes, such as CMV-Luc (data not shown). These data suggest that Ras-mediated activation of either the Raf/MEK/MAP kinase pathway or an additional signaling pathway independent of Raf-1 activation inhibits MyoD-initiated skeletal myogenesis in C3H10T1/2 cells.

Signaling by activated Rac1 or RhoA proteins does not inhibit skeletal myogenesis. The ability of H-Ras 12V,40C to inhibit the myogenic competence of MyoD-expressing C3H10T1/2 cells strongly implicates the activation of Rac and/or Rho family members in this process since the ability of H-Ras 12V,40C to initiate membrane ruffling relies on the function of Rac proteins (16). To test this, C3H10T1/2 fibroblasts were transiently transfected with TnI-Luc, pEM-MyoD, and expression plasmids encoding the constitutively activated forms of the small, monomeric G proteins Rac1 115I and RhoA 63L (17). After 48 h in DM, lysates from the transfected cultures were analyzed for luciferase activity. While control cells transfected with TnI-Luc and pEM-MyoD generated the expected high level of reporter gene activity, C3H10T1/2 cells transfected with TnI-Luc, pEM-MyoD, and either Rac1 115I or RhoA 63L generated levels of luciferase activity equal to or slightly higher than control levels (Fig. 2A). Parallel cultures examined for myogenic competence revealed that the same level of fiber formation occurred in all groups (Fig. 2B), confirming that Rac1 and RhoA expression does not affect MyoD-induced myogenesis. To ensure that Rac1 and RhoA proteins were present in these cells and that MyoD levels were not altered in response to overexpression of these intracellular G proteins, cell lysates again were prepared and analyzed by Western blotting. As predicted, Rac1 and RhoA levels were elevated in transfected cells compared to controls and MyoD levels were not influenced by the overexpression of either protein (Fig. 2C).

Although the data presented in Fig. 2 argue that the failure

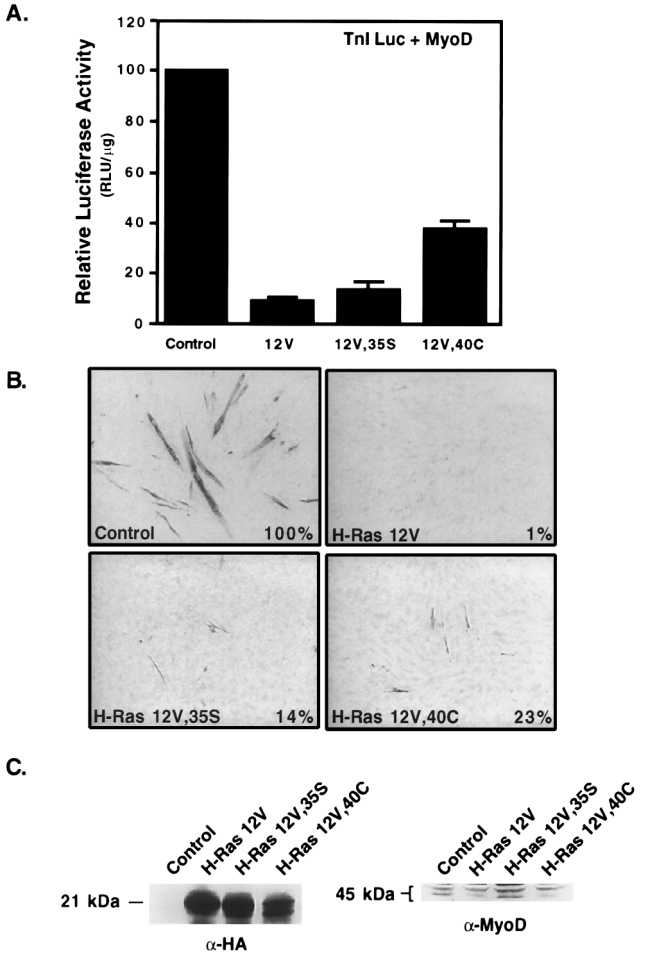

FIG. 1. H-Ras 12V proteins inhibit MyoD-induced myogenesis. (A) C3H10T1/2 cells were transiently transfected with 1.0 μg of TnI-Luc, 0.25 μg of pEM-MyoD, and 0.5 μg of pDCR vector (control), pDCR H-Ras 12V (12V), pDCR H-Ras 12V,35S (12V,35S), or pDCR H-Ras 12V,40C (12V,40C) cDNAs. Following treatment with DM for 48 h, cell extracts were prepared, normalized for protein content, and assayed for luciferase activity as described in Materials and Methods. Luciferase activity (in relative light units [RLU]) is expressed relative to that for the control group, for which the value was set at 100. Each value represents the average of at least three independent transfections. Error bars indicate the standard errors of the means. (B) C3H10T1/2 fibroblasts were transiently transfected with 2.5 μg of pEM-MyoD and 5.0 μg of pDCR (control), pDCR H-Ras 12V (H-Ras 12V), pDCR H-Ras 12V,35S (H-Ras 12V,35S), or pDCR H-Ras 12V,40C (12V,40C) expression plasmids. Following 48 h in DM, the cells were fixed and immunostained for MHC expression as described in Materials and Methods. Representative microscope fields from the indicated experimental groups, viewed and photographed under bright-light conditions, are shown. Ten random fields from each group also were scored for myofiber formation, and the average number of myofibers per field is indicated on the right of each panel as a percentage of the average value obtained for the control group (105 myofibers/field = 100%). The standard errors of the means for the groups are as follows: H-Ras 12V, ±0.4; H-Ras 12V,35S, ±3.7; and H-Ras 12V,40C, ±3.2. Magnification, ×62. (C) C3H10T1/2 cells were transiently transfected with pEM-MyoD and the indicated expression vectors as described for panel B. Following treatment with DM for 48 h, cell extracts were prepared and analyzed by Western blotting. Anti-HA antigen antibodies (α-HA) were used to detect HA-tagged H-Ras proteins, and anti-MyoD antibodies (α-MyoD) were used to detect MyoD proteins.

of Rac1 115I and RhoA 63L to inhibit myogenesis is not due to insufficient levels of protein expression, an additional experiment was performed to examine if the cellular signaling pathway stimulated by activated Rac and Rho proteins is functional in these cells. C3H10T1/2 fibroblasts were transiently cotrans-

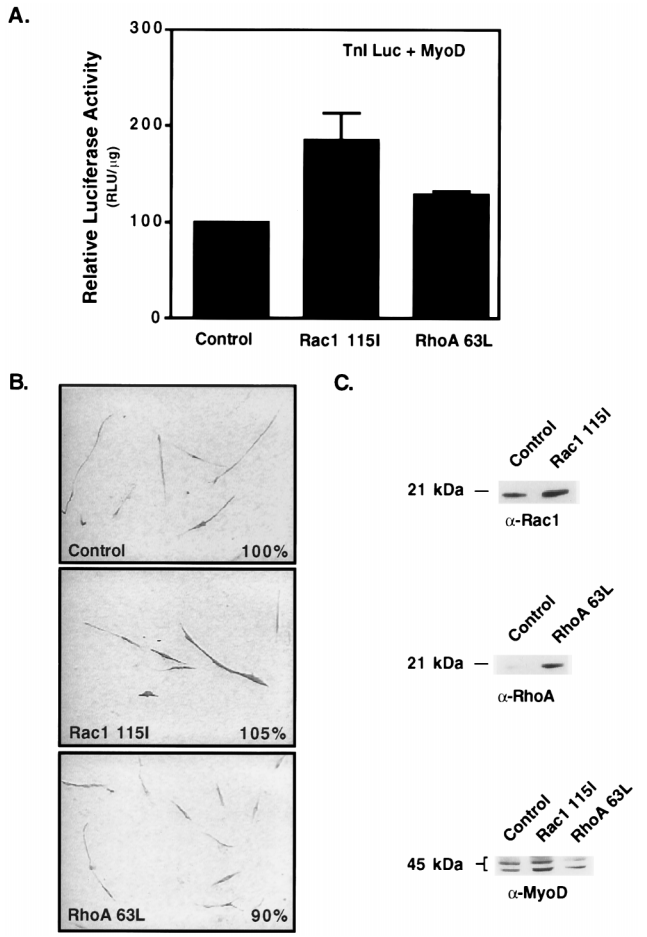

FIG. 2. Activated Rac1 and RhoA proteins fail to block MyoD-induced myogenesis. (A) C3H10T1/2 cells were transiently transfected with 1.0 μg of TnI-Luc, 0.25 μg of pEM-MyoD, and 0.5 μg of pZIPneo vector (control), pZIPRac1 115I (Rac1 115I), or pZIPRhoA 63L (RhoA 63L) cDNAs. Following treatment with DM for 48 h, cell extracts were prepared and the relative luciferase activity for each experimental group was determined as described in the legend to Fig. 1A. RLU, relative light units. (B) C3H10T1/2 cells transiently transfected with 2.5 μg of pEM-MyoD and 5 μg of pZIPneo (control), pZIPRac1 115I (Rac1 115I), or pZIPRhoA 63L (RhoA 63L) cDNAs were maintained in DM for 48 h and then fixed and immunostained for MHC expression as described in Materials and Methods. Each panel shows a representative field from the indicated experimental group. The percent myofiber formation (indicated on the right in each panel) was determined as described in the legend to Fig. 1B. The average myofiber formation in the control group was 51 myofibers/field, with the following standard errors of the means: Rac1 115I, ±2.5; and RhoA 63L, ±12.5. Magnification, ×59. (C) C3H10T1/2 cells were transiently transfected with pEM-MyoD and the expression vectors described for panel B, and protein expression was analyzed by Western blotting as described in the legend to Fig. 1C by using antibodies to Rac1 (α-Rac1), RhoA (α-RhoA), and MyoD (α-MyoD).

fected with SRF2-Luc, a reporter gene used previously to measure activation of the Rac/Rho pathway in cells (14), and expression plasmids encoding H-Ras 12V, H-Ras 12V,40C, Rac1 115I, or RhoA 63L. After 48 h in DM, cell extracts were prepared and luciferase activity was measured. As shown in Fig. 3, all of the test constructs activated expression of SRF2-Luc, with the Rac1 115I and RhoA 63L constructs generating four- and twofold less activity than H-Ras 12V and H-Ras 12V,40C, respectively. While these results demonstrate that each of the G proteins remains functional under differentiation-promoting conditions, the levels of reporter gene activity induced by Rac1 115I and RhoA 63L in C3H10T1/2 cells are lower than those observed in other cell lines (14), suggesting that the traditional Rac1 and RhoA signaling pathways may not be fully functional in these cells. However, the observation that H-Ras 12V,40C induces levels of SRF2-Luc reporter gene activity similar to those of signaling initiated by Rac1 115I and RhoA 63L indicates that the H-Ras 12V,40C-initiated block of myogenesis does not rely on Rac1 or RhoA activation. This conclusion is further supported by results demonstrating that dominant negative Rac1 or RhoA expression plasmids have no effect on the inhibition of TnI-Luc reporter gene activity induced by H-Ras 12V or the H-Ras 12V,40C variant (data not shown). We concluded that activated Rac1 and RhoA do not block myogenesis in this model system and that the Rac/Rho signaling pathway does not play a major role in mediating myogenic repression by the H-Ras 12V protein.

Activation of a Raf-mediated signaling cascade inhibits myogenesis. Perhaps the best-characterized signaling pathway initiated by Ras is the Raf/MEK/MAP kinase cascade. Since H-Ras 12V and H-Ras 12V,35S activate Raf-1 (45) and similarly block myogenic differentiation, we reasoned that a Raf-1-mediated signaling pathway may have a direct effect on terminal differentiation events. To examine the effects of Raf-1 kinase activity on skeletal myogenesis, C3H10T1/2 fibroblasts were transiently transfected with TnI-Luc, pEM-MyoD, and expression vectors encoding wild-type Raf-1 or Raf-1 CAAX, a plasma membrane-localized, constitutively activated variant of human Raf-1 kinase (38). After 48 h in DM, cell extracts were prepared and luciferase activity was measured. Transfection of control cells with pEM-MyoD directed high levels of transcription from the TnI-Luc reporter, as did the transfection of C3H10T1/2 cells with TnI-Luc, pEM-MyoD, and wild-type Raf-1 (Fig. 4A). On the other hand, cells transfected with Raf-1 CAAX failed to demonstrate significant levels of luciferase activity. These observations suggest that constitutive activation of Raf-1 kinase activity by, in this case, localization of the kinase to the plasma membrane mimics the effects of H-

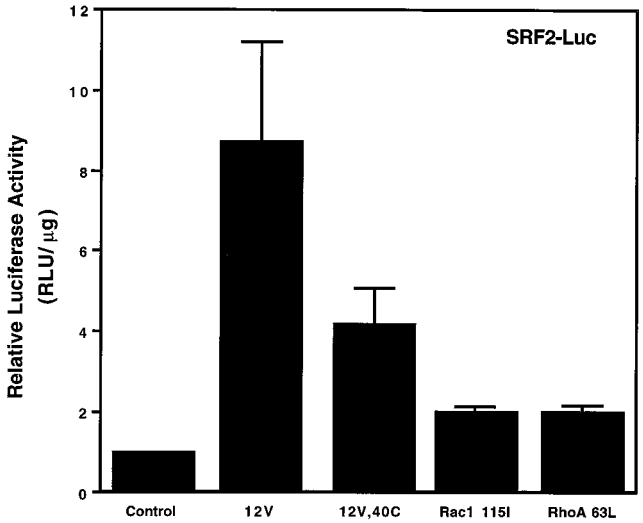

FIG. 3. Rac1 115I and RhoA 63L activate transcription of an SRF-Luc reporter gene. C3H10T1/2 cells were transiently transfected with 1.0 μg of SRF2-Luc and 2.0 μg of pZIPneo or pDCR (control), pDCR H-Ras 12V (12V), pDCR H-Ras 12V,40C (12V,40C), pZIP Rac1 115I (Rac1 115I), or pZIP RhoA 63L (RhoA 63L) cDNAs, and after 48 h in DM, cell extracts were prepared and analyzed for luciferase activity. Activity levels are reported relative to the level of luciferase activity obtained in the control group, for which the value was set at 1.0. Each value is the average of at least three independent transfections. Error bars represent the standard errors of the means. RLU, relative light units.

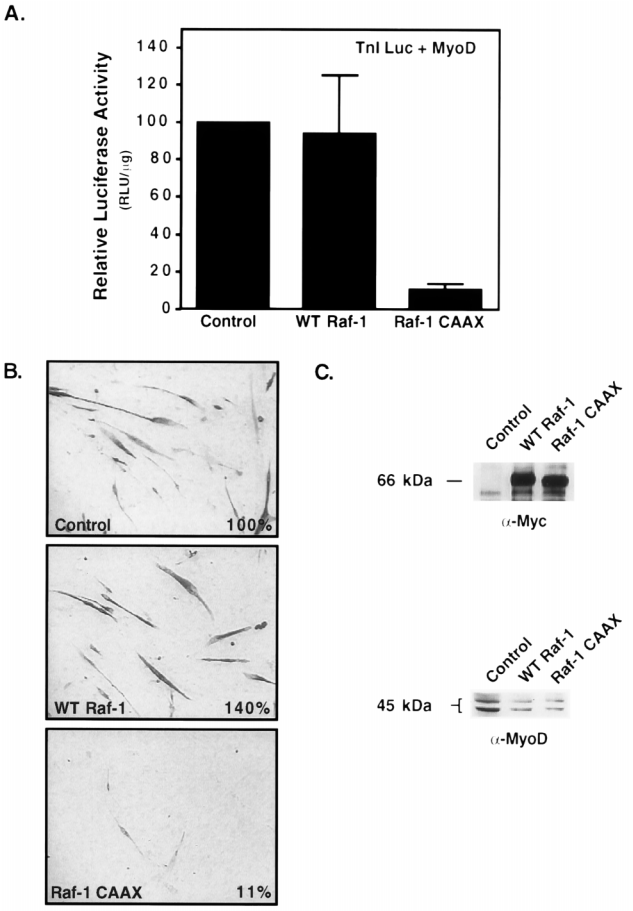

FIG. 4. A constitutively activated Raf-1 protein inhibits MyoD-induced myogenesis. (A) C3H10T1/2 cells were transiently transfected with 1.0 μg of TnI-Luc, 0.25 μg of pEM-MyoD, and 0.5 μg of pMT (control), pMT Raf-1 (WT Raf-1), or pMT Raf-1 CAAX (Raf-1 CAAX) expression plasmids. Following 48 h in DM, cell extracts were prepared and the relative luciferase activity was measured as described in the legend to Fig. 1A and expressed relative to the control group, for which the value was set at 100. RLU, relative light units. (B) C3H10T1/2 cells were transiently transfected with 2.5 μg of pEM-MyoD and 5.0 μg of pMT (control), pMT Raf-1 (WT Raf-1), or pMT Raf-1 CAAX (Raf-1 CAAX) cDNAs, and after 48 h in DM, the cells were fixed and immunostained for MHC as described in Materials and Methods. Each panel shows a representative field from the indicated experimental group. The percent myofiber formation (indicated on the right in each panel) was determined as described in the legend to Fig. 1B. The average myofiber formation in the control group was 83 myofibers/field (100%), and the standard errors of the means were as follows: WT Raf-1, ±22.6; and Raf-1 CAAX, ±2.0. Magnification, ×58. (C) C3H10T1/2 cells were transiently transfected with pEM-MyoD and the indicated constructs, and protein expression was analyzed by Western blotting as described in the legend to Fig. 1C by using anti-Myc (α-Myc) to detect expression of the Myc epitope-tagged Raf-1 proteins and antibodies to MyoD (α-MyoD) to detect the MyoD protein.

Ras 12V and inhibits the ability of MyoD to activate the myogenic program in C3H10T1/2 cells. Immunostaining for MHC in parallel transfected cultures confirmed that Raf-1 CAAX, but not wild-type Raf-1, inhibits the complete myogenic program (Fig. 4B). The inhibition of differentiation was not due to a decrease in MyoD protein accumulation, since comparable levels of MyoD and equivalent levels of Raf-1 were detected in both differentiation-competent (wild-type Raf-1) and differentiation-incompetent (Raf-1 CAAX) groups (Fig. 4C). Once again, expression of control reporter genes in the presence of Raf-1 or Raf-1 CAAX confirmed that these kinases do not function as general inhibitors of transcription activation in C3H10T1/2 cells (data not shown).

Signaling initiated by constitutively active MEK1 does not block skeletal myogenesis. The ability of constitutively active H-Ras and Raf-1 proteins to inhibit myogenesis suggests that Raf-1 activation of MEK1 may mediate this inhibition through the activation of MAP kinase. To examine the effects of MEK1 activity on skeletal myogenesis, C3H10T1/2 cells were transiently transfected with TnI-Luc, pEM-MyoD, and expression plasmids encoding constitutively active MEK1 (MEK E217/E221) or a MEK1 mutant (MEK A221) which is unresponsive to Raf-1 and functions as an interfering protein in cellular transformation assays (6). After 48 h in DM, cell extracts were prepared and luciferase activity was measured. Cells transfected with MEK E217/E221 or MEK A221 exhibited a modest (20%) decrease in TnI-Luc expression compared to control cells (Fig. 5A). A similar level of differentiation also was observed when parallel cultures were stained for MHC accumulation (Fig. 5B). Comparable levels of MyoD were present in each group (Fig. 5C), and the MEK E217/E221 and MEK A221 proteins were expressed well above endogenous levels (Fig. 5C). Thus, we concluded that alteration of MEK1 protein activity, by overexpressing a constitutively activated or an interfering, nonfunctional form of the kinase, has little effect on MyoD-induced terminal differentiation.

Increased MAP kinase activity is not essential for inhibition of myogenesis by Raf-1 CAAX or by H-Ras 12V. Overexpression of constitutively activated Raf-1 inhibits MyoD-induced muscle differentiation in C3H10T1/2 cells. Since the Raf-1 kinase is responsible for the activation of MEK1, which in turn activates MAP kinase (8), elevated levels of MAP kinase activity should be associated with the expression of activated Raf-1 (Raf-1 CAAX), MEK E217/E221, H-Ras 12V, and H-Ras 12V,35S in C3H10T1/2 cells. As a first indication of the relative levels of MAP kinase activity associated with the ectopic expression of these activator proteins in C3H10T1/2 cells, transient-transfection assays were performed with a luciferase reporter gene controlled by five Gal4 binding sites ($Gal4_5$-Luc) and a chimeric activator (Gal4-Elk1) consisting of the Gal4 DNA binding domain and the transcription activation domain of Elk1. Previous studies have shown that the posttranslational modification of the Elk1 transcription activation domain by MAP kinase enhances its transcription activity, providing a convenient measure of relative MAP kinase activity in cells (18). As shown in Fig. 6, expression of Raf-1, Raf-1 CAAX, H-Ras 12V, H-Ras 12V,35S, and MEK E217/E221 generated $Gal4_5$-Luc activities that were significantly higher than those of the control cells. Conversely, Gal4-Elk1 transcriptional activity was not enhanced following expression of the inactive MEK A221 protein or H-Ras 12V,40C. These results confirm that Raf-1, H-Ras 12V, and MEK1 functioned as predicted and that downstream components of the MAP kinase signaling pathway were intact in C3H10T1/2 cells. Furthermore, the fact that expression of H-Ras 12V,40C does not result in MAP kinase activation yet still inhibits myogenesis (Fig. 1), and that MEK E217/E221 activates MAP kinase but does not block myogenesis (Fig. 5), suggests that increased MAP kinase activity is not required for H-Ras 12V, H-Ras 12V,35S, and perhaps Raf-1 CAAX to block skeletal muscle differentiation in this model system.

To further examine the role of MAP kinase activation in the inhibition of myogenic differentiation by molecules such as Raf-1 CAAX and H-Ras 12V,35S, we used PD98059, a chemical inhibitor of MEK1, the immediate upstream activator of MAP kinase (2). The specificity of PD98059 for MEK1 and the parameters of utilizing this compound successfully to modulate

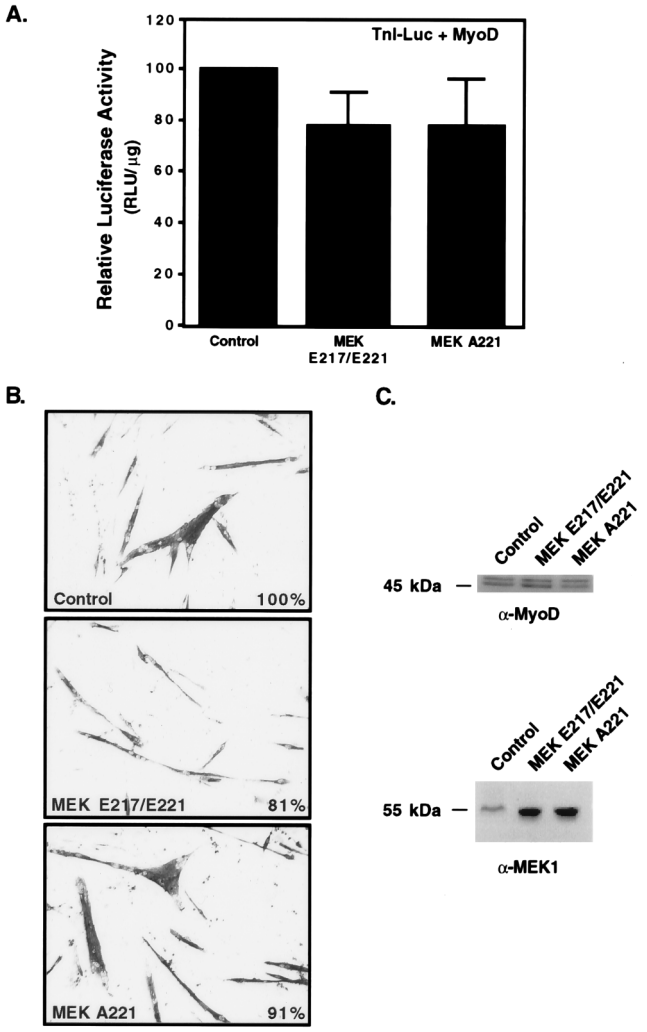

FIG. 5. Constitutively active MEK1 does not significantly alter MyoD-induced myogenesis. (A) C3H10T1/2 cells were transiently transfected with 1.0 μg of TnI-Luc, 0.25 μg of pEM-MyoD, and 0.5 μg of pBABE (control), pBABE MEK E217/E221, or pBABE MEK A221 expression plasmids. Following 48 h in DM, cell extracts were prepared and the relative luciferase activity was measured as described in the legend to Fig. 1A and expressed relative to the control group, for which the value was set at 100. RLU, relative light units. (B) C3H10T1/2 cells were transiently transfected with 2.5 μg of pEM-MyoD and 5.0 μg of pBABE (control), pBABE MEK E217/E221, or pBABE MEK A221 expression plasmids, and after 48 h in DM, the cells were fixed and immunostained for MHC as described in Materials and Methods. Each panel shows a representative field from the indicated experimental group. The percent myofiber formation (indicated on the right in each panel) was determined as described in the legend to Fig. 1B. The average myofiber formation in the control group was 102 myofibers/field (100%), and the standard errors of the means were as follows: MEK E217/E221, ±5.8; and MEK A221, ±9.0. Magnification, ×63. (C) C3H10T1/2 cells were transiently transfected with pEM-MyoD and the indicated constructs, and protein expression was analyzed by Western blotting as described in the legend to Fig. 1C by using anti-MyoD (α-MyoD) to detect the MyoD protein and antibodies to MEK1 (α-MEK1) to detect the MEK1 protein.

MAP kinase activity in cultured cells have been described previously (10, 31). Control studies from our own laboratory have demonstrated that PD98059 treatment of C3H10T1/2 cells under growth- or differentiation-inducing conditions does not decrease cell viability or inhibit transcription of a CMV-*lacZ* reporter gene (data not shown). C3H10T1/2 fibroblasts were transfected with pEM-MyoD or cotransfected with pEM-MyoD and Raf-1 CAAX, H-Ras 12V, H-Ras 12V,35S, and H-Ras 12V,40C. Duplicate plates then were treated with DM or with DM containing 50 μM PD98059. After 48 h, the cultures were fixed and immunostained with an antibody against the muscle-specific MHC protein. As expected (Fig. 1 and 4), cells overexpressing MyoD and H-Ras 12V, H-Ras 12V,35S, H-Ras 12V,40C, or Raf-1 CAAX failed to fully differentiate, as evidenced by the low number of MHC-positive fibers detected in these cultures (Fig. 7). Interestingly, although exposure to the MEK1 inhibitor resulted in a modest, positive effect on the level of MHC staining in all experimental groups, in no case was differentiation scored above 50% relative to controls. The level of rescue was most prominent in the group expressing H-Ras 12V,35S (a twofold increase in the presence of PD98059), yet the absolute level of myogenic differentiation in the treated cells was only 40% of that of the control cultures and essentially equal to the level observed in cells rendered differentiation defective by expression of H-Ras 12V,40C.

It remains formally possible that the incomplete reversal of the nonmyogenic phenotype in the experiments described above results from the inability of PD98059 to block the activation of MAP kinase in C3H10T1/2 cells. To test this hypothesis, C3H10T1/2 cells stably expressing Raf-1 CAAX or each of the H-Ras 12V constructs were maintained in DM with or without the MEK1 inhibitor for 48 h prior to harvest. Total cellular MAP kinase protein was immunoprecipitated from each lysate and assayed for the ability to phosphorylate MBP as described in Materials and Methods. As expected, MAP kinase activity was detected in all experimental groups, with the highest activity observed in the untreated, H-Ras 12V-expressing cultures (Fig. 8). Treatment with the MEK1 inhibitor reduced MAP kinase activity in all PD98059-treated cells. In fact, for groups showing elevated MAP kinase activity in the

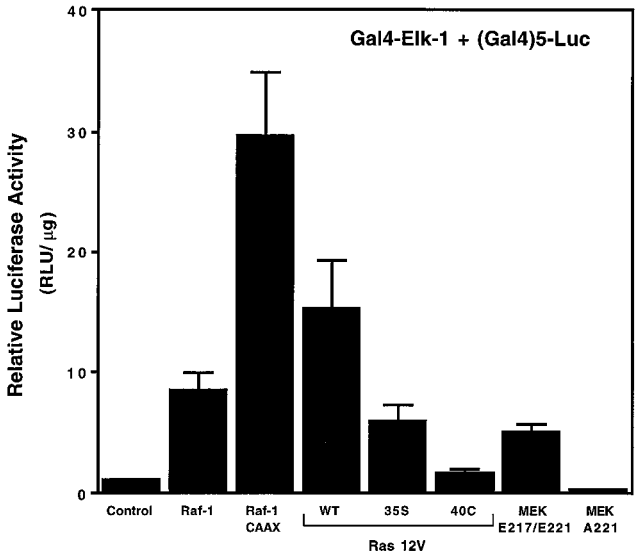

FIG. 6. Constitutively activated Raf-1, H-Ras, or MEK1 enhances transactivation by a Gal4-Elk1 fusion protein. C3H10T1/2 cells were transiently transfected with 1.0 μg of $Gal4_5$-Luc, 0.25 μg of pEM-MyoD, 0.5 μg of Gal4-Elk1, and 0.5 μg of pDCR (control), pMT Raf-1 (Raf-1), pMT Raf-1 CAAX (Raf-1 CAAX), pDCR H-Ras 12V (WT), pDCR H-Ras 12V,35S (35S), pDCR H-Ras 12V,40C (40C), pBABE MEK E217/E221 (MEK E217/E221), or pBABE MEK A221 (MEK A221) expression plasmids. Following 48 h in DM, cell extracts were prepared and analyzed for luciferase activity. Activity levels are reported relative to the level of luciferase activity obtained in the control group, for which the value was set at 1.0. Each value is the average of at least three independent transfections. Error bars represent the standard errors of the means. RLU, relative light units.

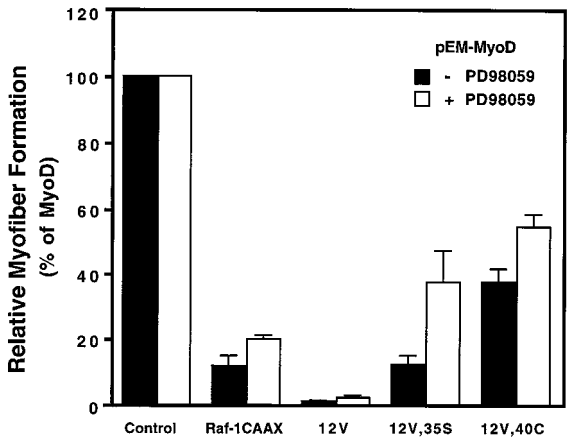

FIG. 7. Treatment with the MEK1 inhibitor PD98059 does not restore myogenic competence to MyoD-transfected C3H10T1/2 cells expressing activated Raf-1 or H-Ras proteins. C3H10T1/2 cells were transiently transfected with 2.5 μg of MyoD and 5.0 μg of pDCR (control), pMT Raf-1 CAAX (Raf-1 CAAX), pDCR H-Ras 12V (12V), pDCR H-Ras 12V,35S (12V,35S), or pDCR 12V,40C (12V,40C) DNAs; each group was treated in parallel with DM with or without 50 μM PD98059, a MEK1-specific inhibitor. Forty-eight hours following transfection, the cells were fixed and immunostained to detect expression of the muscle-specific MHC protein. All groups were examined for the relative frequency of myofiber formation as described in the legend to Fig. 1B, and the percent myofiber formation is reported relative to the number of myofibers in the control groups, for which the values were set at 100 (121 myofibers/field [untreated] and 93 myofibers/field [treated]). Error bars represent the standard errors of the means.

absence of the inhibitor, addition of the inhibitor reduced MAP kinase activity to levels equal to or below those of the MyoD control groups, which were fully competent for differentiation. These studies demonstrate that while the MEK1 inhibitor functions as expected, reducing MAP kinase activity is not sufficient to restore the myogenic phenotype in H-Ras- or Raf-1 CAAX-expressing cells. In addition, since the inhibition of MyoD-induced myogenesis by H-Ras 12V is not duplicated by the activation of Rac1, RhoA, or MEK1, our data strongly suggest that an additional pathway, distinct from the activation of MEK/MAP kinase or Rac and Rho, is involved in the inhibition of skeletal muscle differentiation by H-Ras 12V and Raf-1 CAAX.

DISCUSSION

Constitutive activation of Ras proteins has been shown to inhibit the induction of skeletal myogenesis in culture (11, 19, 20, 22, 30). In the case of H-Ras 12V, recent work from our laboratory has demonstrated that while the antimyogenic effect of Ras can be assessed by monitoring the inability of a coexpressed MRF, such as MyoD or MRF4, to induce muscle fiber formation in C3H10T1/2 cells, the target of Ras inhibition is not the MRF protein itself (19). Activation of Ras does not negatively impact the ability of the MRFs to bind to DNA, nor does it adversely affect the ability of the MRFs to activate transcription of a synthetic reporter gene containing only MRF binding sites (19). Thus, Ras signal transduction blocks myogenesis by targeting an accessory molecular process (or processes) that is essential for MRF function to be translated into the production of a fully differentiated skeletal muscle cell. This molecular process may act upstream of the MRFs or, most likely, operate in parallel to the MRFs. However, attempts by a number of laboratories to identify MRF cofactors whose regulation could account for the observed behavior of the MRFs in the above assays have not been fruitful.

An additional approach to further define the molecular mechanism(s) underlying the Ras-mediated inhibition of skeletal muscle differentiation is to examine the role of signaling molecules immediately downstream of Ras. The recent explosion of information with regard to the components of the Ras signal transduction pathway in cells (8) and the characterization of Ras derivatives bearing single amino acid substitutions that ablate activation of specific downstream Ras effector molecules (16, 45) have made it possible to identify the signaling events responsible for many Ras-associated cellular phenotypes. In the case of oncogenic transformation by Ras, there is a dependence on the activation of the Raf/MEK/MAP kinase cascade and on the ability of Ras to induce alterations in cellular architecture through the activation of Rac and Rho. Utilizing the same molecular reagents as have been used for transformation studies, we have made the striking observation that activation of distinct Ras signaling pathways, one pathway which results in increased levels of MAP kinase activity and a second pathway which does not, inhibits the biochemical and morphological differentiation of C3H10T1/2 fibroblasts expressing MyoD. Our initial interpretation of these data was that activation of either the Rac/Rho or the Raf/MEK/MAP kinase cascade may be sufficient to inhibit skeletal muscle precursors from differentiating. However, follow-up studies have produced data supporting an alternative explanation, namely, that an additional, yet-to-be-identified Ras-stimulated signaling pathway is largely responsible for blocking myogenesis in this model system (Fig. 9).

The behavior of the H-Ras 12V,40C molecule was critical to our arriving at this conclusion. While the cellular effectors that bind directly to this transformation-defective Ras variant have not been identified, it has been shown that H-Ras 12V,40C expression modifies cellular architecture in a Rac/Rho-dependent fashion yet does not stimulate cell growth via activation of the Raf/MEK/MAP kinase pathway (16). Assays in which the levels of MAP kinase activity were examined in C3H10T1/2 cells expressing H-Ras 12V,40C (Fig. 6 and 8) confirm the latter observation. Expression of H-Ras 12V,40C results in a 60% inhibition of MyoD-induced myogenesis, which is not reproduced by expression of constitutively activated Rac1, constitutively activated RhoA, or both activated proteins (Fig. 2 and data not shown). Since these constitutively active proteins are produced (Fig. 3) and are able to function as expected in C3H10T1/2 cells (Fig. 2), we suggest that a Ras signaling path-

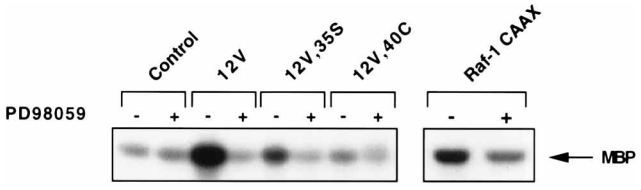

FIG. 8. PD98059 reverses the increase in MAP kinase activity induced by constitutive activation of H-Ras or Raf-1 in C3H10T1/2 cells. Stable C3H10T1/2 cell lines expressing the indicated H-Ras or Raf-1 CAAX constructs were generated as described in Materials and Methods and maintained in DM (with or without 50 μM PD98059) for 48 h. MAP kinase was immunoprecipitated from cell extracts prepared from each group and assayed for activity by using [γ-^{32}P]ATP and MBP as a substrate. Reactions were subjected to SDS-PAGE and analyzed by autoradiography. While cells expressing H-Ras 12V, H-Ras 12V,35S, or Raf-1 CAAX protein displayed elevated levels of MAP kinase activity, treatment with PD98059 reduced the activity to control levels or to levels found in cells expressing an H-Ras variant (H-Ras 12V,40C) which does not activate the Raf/MEK/MAP kinase pathway (see the text for details).

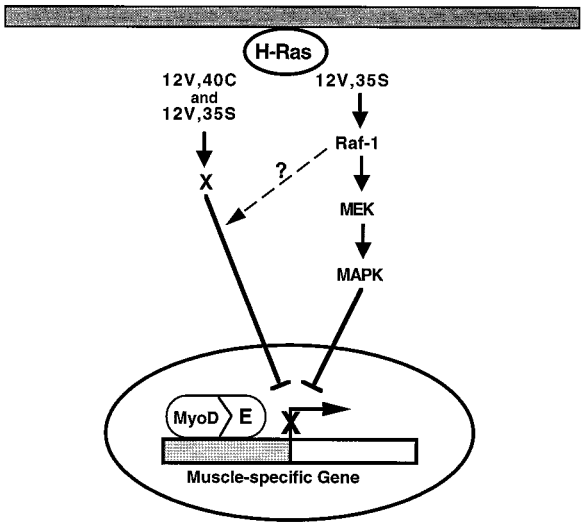

FIG. 9. Model for the inhibition of skeletal myogenesis by signaling pathways activated by oncogenic Ras. The data presented in this paper indicate that enhanced activation of the Raf/MEK/MAP kinase cascade by H-Ras 12V,35S, while contributing to the H-Ras-mediated inhibition of skeletal myogenesis, is not solely responsible for the observed effect. Synergistic activation of another signaling pathway by H-Ras 12V,40C (and almost certainly by H-Ras 12V,35S) is required. A novel effector or set of effectors (X) is activated by all three H-Ras 12V proteins. Signaling through X occurs independently of Rac and Rho activity, yet Raf-1 signaling may converge at, function downstream of, or operate in parallel with X. The direct target(s) of negative regulation by these pathways is not known, yet the ultimate target is the MRF-E protein transcription complex (MyoD>E), which mediates the differentiation of skeletal muscle via the transcriptional activation of muscle-specific genes and whose myogenic function is blocked completely in H-Ras 12V-expressing cells.

way, distinct from the Rac/Rho and Raf/MEK/MAP kinase pathways, is responsible for myogenic inhibition by H-Ras 12V,40C.

In order to demonstrate fully that a third signaling pathway is essential for the inhibition of myogenesis by Ras, it was necessary to establish that the H-Ras 12V proteins stimulating Raf-1, MEK1, and, subsequently, MAP kinase activity in C3H10T1/2 cells do not depend solely on elevated MAP kinase activity to inhibit myogenesis. The documented role of MAP kinase activation in intracellular signaling triggered by the exposure of cells to mitogens, growth factors, and oncoproteins, many of which have been shown to inhibit myogenesis (1), strongly suggests that an increase in intracellular MAP kinase activity would adversely affect skeletal myogenesis. However, the studies presented in this paper demonstrate that activation of MAP kinase plays a minimal role in the inhibition of myogenesis by H-Ras 12V proteins. Overexpression of constitutively active MEK1, while generating levels of Gal4-Elk1 and $Gal4_5$-Luc activity that are similar to those exhibited by the myogenic inhibitor H-Ras 12V,35S (Fig. 6), did not cause a significant reduction in muscle-specific gene expression (Fig. 5). In addition, treatment of MyoD-expressing C3H10T1/2 cells with the chemical MEK1 inhibitor PD98059 (2) was unable to reverse the block in myogenesis induced by a coexpressed, activated Ras protein (Fig. 7). These results are supported by previous studies showing that the PD98059-induced reduction of MAP kinase activity in 23A2 mouse myoblasts does not block differentiation of wild-type cells or restore differentiation in a Ras-expressing 23A2 cell line (44). The latter result is made even more intriguing by the observation that PD98059 arrests the growth of 23A2 Ras-expressing cells (44), and we have noted that PD98059 similarly represses the continued growth imposed on C3H10T1/2 cells by expression of the H-Ras 12V or H-Ras 12V,35S protein (unpublished observations). We have concluded from these studies that the induction of MAP kinase activity by H-Ras 12V is essential for continued cell growth, and while growth may play a limited role in the absolute levels of myogenic inhibition achieved by an activated Ras protein (compare H-Ras 12V,35S to H-Ras 12V,40C [Fig. 7]), it is clear that a MAP kinase-independent pathway functions as the major pathway in the Ras-induced inhibition of skeletal muscle differentiation.

The data in this paper strongly imply that a Ras-stimulated pathway, distinct from the well-characterized Raf/MEK/MAP kinase and Rac/Rho pathways, is the major pathway responsible for the inhibition of myogenesis by H-Ras 12V. Raf-1 also may signal via this novel pathway since PD98059 treatment does not reverse the inhibition of myogenesis by Raf-1 CAAX (Fig. 7). We have performed cotransfection experiments with H-Ras 12V,35S and H-Ras 12V,40C and have observed that these proteins do not act synergistically to inhibit the myogenic program (unpublished observations), suggesting that they may function to activate a common downstream signaling intermediate (Fig. 9). By utilizing various library screening strategies and in vitro binding techniques, additional Ras effector molecules recently have been identified, some of which have well-defined roles in signaling (e.g., RalGDS and PI3 kinase) (34, 36, 40, 46), while others have yet to be assigned specific positions within known signaling cascades (e.g., KSR and AF6) (9, 43). Future studies will be aimed at establishing whether activation of one, or several, of these additional Ras effector molecules defines the mechanism through which H-Ras 12V blocks myogenesis.

Substantial progress in characterizing the molecules responsible for the differentiation of muscle precursor cells in vitro and in vivo has provided researchers with a model system to investigate how the fate of cells becomes restricted as development progresses. Equally important to our understanding of fate restriction is understanding how the instructions specifying fate are overridden by the exposure of cells to environmental stimuli or by the mutational activation of signaling molecules whose misexpression disrupts differentiation and often results in disease. For over a decade, efforts to catalog the various inhibitors of skeletal myogenesis have fueled the opinion that the agents negatively controlling myogenesis are those that induce cells to grow. The first evidence to refute this opinion was the finding that exposure of growth-arrested myoblasts to FGF-2 inhibits differentiation without promoting cell cycle reentry (37). Now, the data in this paper strongly indicate that oncogenic Ras inhibits differentiation by a molecular mechanism that is distinct from its ability to reorganize cellular architecture or stimulate cell proliferation. Future advances in analyzing Ras signaling events with skeletal muscle as a model system will establish the components of alternative Ras signaling pathways and undoubtedly reveal new information on how developmental processes are fine-tuned in vivo.

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