Inhibition of Myogenesis by the H-ras Oncogene: Implication of a Role for Protein Kinase C

Tushar B. Vaidya,* Crystal M. Weyman,‡ Dorothy Teegarden,§ Curtis L. Ashendel,‡ and Elizabeth J. Taparowsky*

*Department of Biological Sciences, ‡Department of Medicinal Chemistry and Pharmacognosy, and §Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

Abstract. Expression of the oncogenic form of H-ras p21 in the mouse myogenic cell line, 23A2, blocks myogenesis and inhibits expression of the myogenic regulatory factor gene, MyoD1. Previous studies from a number of laboratories have demonstrated that the activation of ras p21 is associated with changes in phospholipid metabolism that directly, or indirectly, lead to elevated levels of intracellular diacylglycerol and the subsequent activation of protein kinase C (PKC). To assess the importance of PKC activity to the ras-induced inhibition of skeletal myogenesis, we examined the levels of PKC activity associated with the terminal differentiation of wild-type myoblasts and with the differentiation-defective phenotype of 23A2 ras cells. We demonstrate that there is a 50% reduction in PKC activity during normal myogenesis and that PKC activity is required for myoblast fusion, but not for the transcriptional activation of muscle-specific genes. In contrast, we found that the differentiation-defective 23A2 ras cells possess two- to threefold more PKC activity than wild-type myofibers and that reducing the PKC activity in these cultures does not reverse their non-myogenic phenotype. On the other hand, if PKC activity is downregulated in 23A2 cells before the expression of activated ras p21, myogenesis is not inhibited. These results suggest that activated ras p21 relies on a PKC-dependent signal transduction pathway to initiate, but not to sustain, its negative effects on 23A2 skeletal myogenesis and underscore the potential importance of PKC activity to the proper control of skeletal muscle differentiation.

The inhibition of MyoD1 gene expression by the ras oncogene is a convenient nuclear marker for investigating further the signal transduction pathways mediating the ras-induced inhibition of myogenesis. The human H-ras gene encodes a plasma membrane associated protein, p21, that resembles eukaryotic G-proteins (reviewed in reference 4). In a variety of mammalian cell types, expression of a mutated, oncogenic form of ras p21 leads to a constitutive increase in intracellular diacylglycerol (18, 24, 28, 39, 50),
which serves as an endogenous activator of the serine-threonine protein kinase C (PKC) \(^1\) (reviewed in references 34, 35). Activation of PKC also can be achieved experimentally by treating cells with phorbol ester tumor promoters (reviewed in references 34, 35) and in several systems, phorbol esters can duplicate or enhance the physiological effects of ras oncogene expression (12, 22, 47, 48). Since MyoD1 is a nuclear phosphoprotein that regulates its own gene expression (44, 45), it is intriguing to speculate that ras-induced alterations in PKC activity may be important to the transcriptional regulation of MyoD1 gene expression and thus important to the control of skeletal myogenesis.

In this study, we have investigated the role of PKC in the normal differentiation of 23A2 myoblasts and in the non-myogenic phenotype of 23A2 ras cells. We demonstrate that the differentiation-defective ras cultures exhibit two to three times more PKC activity than wild-type cells and that prolonged treatment with the phorbol ester tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA), effectively downregulates PKC in both cell types. While TPA treatment adversely affects myocyte function, it does not inhibit the expression of muscle-specific genes in wild-type 23A2 cells or restore myogenesis in the 23A2 ras cultures. Interestingly, the initial response of 23A2 myoblasts to activated ras p21 relies on PKC activity, since downregulating PKC with TPA before the expression of the ras oncogene results in a level of myogenic differentiation that approaches wild-type controls. We conclude from these studies that PKC activity is essential for ras p21 to initiate its negative effects on skeletal myogenesis and MyoD1 gene expression, but that once established, continued high levels of PKC activity are not required for the differentiation-defective ras phenotype to be maintained. These results suggest a critical role for PKC in the establishment of the ras-induced inhibition of 23A2 myogenesis and thus in the regulatory mechanisms controlling MyoD1 gene expression in this cell line.

### Materials and Methods

#### Cell Culture

The mouse myogenic cell line, 23A2, (25), was maintained on gelatin-coated dishes in BME (basal modified Eagle's medium; Gibco Laboratories, Grand Island, NY) supplemented with 15% fetal bovine serum and 100 U/ml penicillin and 100 Ag/ml streptomycin (P/S). Terminal differentiation was induced in subconfluent 23A2 cultures by replacing the growth medium with a defined medium, ITS (52), for 2 d. For experiments in which fusion was scored, enhanced morphological differentiation of the cultures was obtained by treating subconfluent 23A2 monolayers with F12 medium (Gibco Laboratories) supplemented with 15% horse serum, 100 mM CaCl\(_2\), and P/S for 4 d. The 23A2 pTi2492 cell line is a subclone of 23A2 pT2492 (26) and is transfected stably with the oncogenic form of the human H-ras gene. For all experiments, the culture conditions for the 23A2 pTi2492 cells were the same as those described for the parental 23A2 myoblast cell line.

#### Transfections

23A2 myoblasts were transfected with calcium phosphate precipitates as described previously (27, 14) with the following modifications. For stable transfections, 5 \(\times\) 10\(^5\) cells were seeded per 100-mm dish. On the next day precipitates containing 30 ng of pKOneo (43) and 300 ng of either pB3H (42) or pT24 (42) were added to each dish. After 5-6 h, the precipitates were removed and replaced with fresh growth medium. 24 h later, each plate of cells was split 1:10 into growth medium supplemented with an active concentration of 400 \(\mu\)g/ml of G418 (geneticin; Gibco Laboratories). After colonies had formed (>15 d), cultures were treated with F12 differentiation-inducing medium, fixed, and stained with Giemsa (EM Diagnostic Systems, Inc., Gibbstown, NJ) (25). Percent differentiation was determined by microscopic examination and represents the number of colonies displaying discernable myofibers over the total number of colonies examined. For experimental groups treated with TPA or the nonfunctional TPA analogue 4a-phorbol 12-myristate 13-acetate (4a-PMA) (L. C. Services Corporation, Woburn, MA), a 2 ng/ml solution in ethanol (100%) was used to supplement the growth or differentiation medium. For transient transfections, 1 \(\times\) 10\(^5\) 23A2 myoblasts or 23A2 pT2492 cells were seeded per 100-mm dish. After 2 d, precipitates containing 5 \(\mu\)g of 3 \(\times\) TRE CAT (2) and 5 \(\mu\)g of the reference plasmid, RSVlacZ (23), were added to each dish. 4 h later, the cells were shocked osmotically for 2 min with 5 ml of 20% glycerol in BME and then immediately refed fresh growth medium. After 48 h, protein extracts were prepared as described previously (14) and stored at -20°C until evaluated for CAT activity (see below). 23A2 myoblast transfections were performed exactly as described above with the exception that the cells were seeded at 4 \(\times\) 10\(^5\) cells per 100-mm dish and were treated with ITS differentiation-inducing medium after the glycerol shock.

#### CAT Assays

Cell extracts were assayed for chloramphenicol acetyltransferase (CAT) activity as described previously (14). The activities reported represent the percent conversion of chloramphenicol to the acetylated form averaged from two independent transfection experiments. Each assay was maintained within the linear range of CAT activity. To control for the efficiency of transfection, the cell extracts were normalized with respect to the activity of the cotransfected \(\beta\)-galactosidase gene (RSVlacZ) as described in Nielsen et al. (33).

#### Creatine Kinase Assays

Cell extracts from ITS-treated 23A2 and 23A2 ras cells were assayed for the muscle isoform of creatine kinase (MCK) as described previously (26). Each value for MCK activity represents the mean of two independent experiments and has been adjusted for the background level of MCK activity measured in control 23A2 myoblast extracts.

#### Immunocytochemistry

Cultures were fixed and immunocytochemically stained as described previously (27) using Vectastain ABC reagents (Vector Labs, Burlingame, CA) and MF-20, a mouse monoclonal antibody that is specific for the skeletal myosin heavy chain proteins (3). The stained cultures were examined microscopically under bright light conditions at 25× and the percentage of cells expressing myosin heavy chain protein was determined by dividing the number of nuclei in myosin-positive cells by the total number of nuclei counted in a given field. Myofiber formation is expressed as the percentage of total nuclei contained within myosin-positive cells having two or more nuclei. To arrive at these determinations, two independent experiments were performed in which at least five randomly chosen microscope fields, each containing approximately 100 nuclei, were examined for each experimental group.

#### Northern Blot Hybridizations

Total RNA was isolated from cultures using the method of Chomczynski and Sacchi (7). 15 \(\mu\)g of RNA from each sample were electrophoresed through a 1% formaldehyde-agarose gel and transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) as described previously (46). Filters were prehybridized at 65°C in 6× SSC (20× SSC: 3 M NaCl, 300 mM Na-Citrate, pH 7.0), 20 mM Tris, pH 7.5, and 10× Denhardt's solution for 2-4 h and in 6× SSC, 20 mM Tris, pH 7.5, 0.3% SDS containing 100 \(\mu\)g/ml heat-denatured salmon sperm DNA for an additional 2 h. Radioactive probes, including the 2.9-kb SstI ras-specific fragment from pT24 (15), the 1.2-kb EcoRI troponin I-specific fragment from cM113aR (27) and the 1.8-kb EcoRI MyoD1-specific fragment from pEMClIIs (11), were labeled, using \(\alpha\)-32P dCTP (sp act 3000 Ci/\(\mu\)mol; Amersham Corp., Arlington Heights, IL) and the oligolabeling procedure.
of Feinberg and Vogelstein (16, 17). Denatured probes (sp act > 1 × 10^6 cpm/μg) were added directly to the filters in the second prehybridization solution and incubated at 65°C for 12–18 h with gentle agitation. The filters were washed in 0.1× SSC, 2 mM EDTA and 0.1% SDS at room temperature for 10 min and at 68°C for 2 h with one change of buffer. To visualize the hybridization signals, the filters were exposed to XAR film (Kodak) at −80°C with an intensifying screen for 12–24 h.

**Extraction of PKC from Cells**

For the determination of PKC activity in proliferating 23A2 or 23A2 ras cells, cultures were seeded in growth medium at 4 × 10^5 cells per 75-cm² flask and extracts were prepared after 48 h. To obtain myofibroblast extracts, cultures were seeded in growth medium at 4 × 10^5 cells per 75-cm² flask on day 0, treated with ITS medium on day 2, and extracts were isolated on day 4. Cultures were washed twice with cold (4°C) PBS (15 mM NaCl, 8.7 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, pH 7.5), once with cold 7.5% sucrose in 20 mM Tris, pH 7.4, and permeabilized for 90 s with 1 ml of cold lysis buffer (20 mM Tris, pH 7.4, 5 mM EDTA, containing 500 μg/ml digitonin, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin). Cytosolic extracts were collected after this permeabilization treatment. The remaining cell debris was scraped in 1 ml of cold lysis buffer containing 2% CHAPS; disrupted by sonication for 5 s using a Sonifier Ultrasonicator (Heat Systems, Ultrasonics, Inc., Plainview, NY) and cleared by centrifugation in a rotor (SS34 rotor; Sorvall Instruments, Inc., Newton, CT) at 20,000 rpm for 20 min at 4°C. The supernatants isolated by this procedure represent membrane extracts.

**PKC Assays**

PKC activity was measured by the in vitro kinase assay as described previously (49). Briefly, 5–10 μg of cytosolic or membrane extracts were incubated for 10 min at 37°C in 20 mM Tris, pH 7.4, 10 mM MgCl₂, 0.5 mM CaCl₂, 10 mM p-nitrophosphosphate, 20 μM γ-32P ATP (sp act 0.2–1.0 Ci/μmol) containing 80 μg/ml phosphatidylinerse and 240 μg/ml lysine-rich histone HI (Sigma Chemical Co., St. Louis, MO). To assess the contribution of kinases other than PKC to the activity of each extract, duplicate samples were assayed in the presence of 1 mM EDTA and 15 mg/ml CHAPS, in the absence of CaCl₂ and phosphatidylserine. All reactions were terminated by spotting 50-μl aliquots on phosphocellulose filter paper (P81; Whatman Inc., Clifton, NJ). The filters were washed three times in water, dried, and the amount of 32P incorporated into histone determined by scintillation counting. PKC activity is calculated as picomoles of 32P incorporated into histone per minute per milligram protein extract. Although cytosol and membrane extracts were assayed separately, the PKC activities reported represent the total activity (cytosol plus membrane) measured for each experimental group.

**Immunoblots**

Equal volumes of cytosol and membrane extracts were pooled from each experimental group and assays were performed on 66 mg of total cell homogenate. Samples were denatured in 5 mg/ml SDS containing 1% mercaptoethanol and electrophoresed through a discontinuous 10% denaturing polyacrylamide gel by the method of Laemmli (29). The proteins were transferred electrophoretically to nitrocellulose overnight at 4°C. The filters were blocked with 5% Carnation Instant Milk in TNS (15 mM Tris, pH 7.4, 145 mM NaCl) for 10–20 min at room temperature and then incubated with anti–rat brain PKC egg yolk antibodies (49) in incubation buffer (TNS containing 1% BSA, 0.5% sodium azide, and 5% Carnation Instant Milk) for a minimum of 2 h. The filters were washed for a total of 25 min in five changes of TNS containing 1% Tween 20, reblocked with 5% milk in TNS for 5 min, and then reacted with rabbit anti–chicken egg yolk antibodies in incubation buffer for at least 2 h. The filters were washed again in 1% Tween 20 in TNS as described above and incubated with 20 μCi of 35S–protein A (sp act >1,000 Ci/mmol; ICN Biochemicals, Irvine, CA) for 6 h. The cells were rinsed with PBS, scraped in 1 ml of PBSTDS (10 mM sodium phosphate, pH 7.25, 0.9% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% sodium azide) and lysed by passing the suspension through a 21-gauge needle five times. The lysate was cleared by centrifugation at 100,000 rpm in a rotor (TLA 100.3; Beckman Instruments, Inc., Palo Alto, CA) for 5 min at 4°C. 4 × 10^6 cpm of each extract were incubated overnight at 4°C with or without the pan-reactive anti–ras p21 monoclonal antibody, Y13-259 (prepared as described below), and then incubated with 20 μg of coated protein A–Sepharose (prepared as described below) at 4°C for 4–6 h. After five washes with 1 ml of PBSTDS, the precipitates were resolved on a 12.5% denaturing polyacrylamide gel (29). Molecular weight standards were visualized by staining with Coomassie brilliant blue and then the gel was treated with Enhance (DuPont Corp., Wilmington, DE), dried and exposed to XAR film (Kodak) at room temperature. To quantitate ras p21 in each sample, portions of the gel corresponding to the signals were excised, dissolved in a 95:5 (vol/vol) mixture of 30% H_2O_2 and NH_4OH, and counted in a scintillation counter. Background counts from lanes precipitated without the Y13-259 antibody were subtracted from each sample and the remaining counts expressed as cpm/mg protein. Protein concentration was determined in parallel extracts not exposed to radioactivity. The values reported in Fig. 7 B represent the average of three independent experiments.

Precoated protein A–Sepharose was prepared by incubating 1 μg of protein A–Sepharose (Sigma Chemical Co.) overnight at 4°C with 2 μg of cell homogenate and 0.7 μg of rabbit anti–rat antibody (Sigma Chemical Co.) in the presence of leupeptin (1 μg/ml), antipain (2 μg/ml), benzamidine (10 μg/ml), 1% aprotinin, and 1 mM PMSF. The coated Sepharose was rinsed five times with PBSTDS and resuspended as a 1:1 slurry in PBSTDS.

To obtain the Y13-259 antibody, 259 rat hybridoma cells (20) were grown in DMEM supplemented with 10% fetal bovine serum, 20 mM Hepes, and P/S. The medium was collected after 3–5 d, centrifuged to remove the cells, and the antibody precipitated with 50% ammonium sulfate. The precipitate was resuspended in PBS and dialyzed against PBS before use.

**Results**

**Differentiation-defective ras Myoblasts Exhibit Elevated Levels of Protein Kinase C Activity**

Expression of the human H-ras oncogene in the mouse myogenic cell line, 23A2, inhibits both the morphological and the biochemical differentiation of the cells (26). This block in differentiation appears to be associated directly with the downregulation of the myogenic regulatory factor gene, MyoD, since introducing a constitutively expressed MyoD cDNA into 23A2 ras cells restores myogenic competence in these cultures (26). To dissect further the biochemical pathways through which ras p21 inhibits myogenesis and MyoD gene expression, we examined differentiation-defective ras myoblasts for altered levels of the calcium- and phospholipid-dependent protein kinase, PKC. Cell extracts were prepared from proliferating 23A2 myoblasts, from proliferating 23A2 cells stably transfected with the human H-ras oncogene (23A2 pT249a2), and from cultures of 23A2 and 23A2 pT249a2 cells that had been induced to differentiate by treatment with the serum-free medium, ITS (52). Although wild-type 23A2 myoblasts exhibit extensive differentiation when induced with ITS, 23A2 pT249a2 cells do not fuse and do not express skeletal muscle-specific genes (26). Examination of PKC activity in these cells revealed that proliferating 23A2 ras cells contained ~40% more PKC activity than extracts from wild-type 23A2 myoblasts (Fig. 1). After treatment with ITS, PKC activity decreased by 50% in the fully differentiated 23A2 myoblast cultures and by 25% in the differentiation-defective 23A2 ras cells, with the ras cultures retaining a twofold higher level of PKC activity than wild-type
PKC Is Downregulated in 23A2 and 23A2 ras Cells After Treatment with TPA

The elevated level of PKC activity associated with ras oncogene expression in 23A2 cells could be due to an increased rate of PKC protein synthesis, the expression of additional PKC isozymes, or to a decrease in the efficiency of PKC protein degradation. To begin investigating these possibilities, in vitro kinase assays and Western blot analyses were performed to measure PKC activity and PKC protein levels in 23A2 and 23A2 pT249a2 cultures that had been treated with increasing concentrations of the phorbol ester tumor promoter, TPA. As reported previously, exposure of a variety of cell types to TPA causes a transient increase in PKC activity which is followed by the downregulation of the activated enzyme via a host cell proteolytic pathway (reviewed in references 34, 35). Thus, if the transfection of 23A2 cells with activated ras p21 elevates intracellular PKC activity by inhibiting the pathway of PKC degradation, or by stimulating production of PKC isozymes that are resistant to the effects of phorbol esters (9), the PKC pool in 23A2 cells should be downregulated by TPA more efficiently than the PKC pool in 23A2 ras cells.

For these studies, 23A2 and 23A2 pT249a2 cultures were treated with differentiation-inducing medium supplemented with concentrations of TPA between 1 nM and 1 μM. After 48 h, cell extracts were prepared and PKC activity was measured by in vitro kinase assays. As shown in Fig. 3, 1 and 10 nM TPA did not affect PKC activity levels in either cell type. The ras cultures maintained a threefold higher level of PKC activity than the wild-type 23A2 cells. After treatment with 100 nM TPA, PKC activity dropped by 54% in the 23A2 cultures and by 43% in the 23A2 ras cells. Treatment with 1 μM TPA essentially eliminated PKC activity in both cell types since the treated 23A2 and 23A2 pT249a2 cultures retained only 1 and 8% of the PKC activity measured in the untreated control cultures, respectively. These results dem-
ter TPA treatment resulted from proteolysis of the enzyme, Vaidya et al. Role of PKC in Skeletal Muscle Development.

With concentrations of TPA that produced a decrease in PKC rat brain PKC (49) . As shown in Fig. 4, treatment of the cells with a chicken polyclonal antiserum prepared against total acrylamide gel, blotted to nitrocellulose, and then reacted each extract were electrophoresed through an SDS-poly-

had to be treated for 48 h with ITS medium containing 0 nM, or 1 μM TPA. Equal amounts of total protein from each extract were treated with differentiation-inducing medium supplemented with concentrations of TPA ranging from 1 nM to 1 μM. The PKC activity in the cell extracts prepared from each culture was measured as described in Materials and Methods. Each value represents the average of four independent determinations, with the exception of the 500 nM 2342-ras group which was assayed twice. The high and low values for each group of determinations are indicated by the range bars.

Figure 3. PKC activity in TPA-treated 2342 and 2342-ras cells. 2342 cells (△) and the differentiation-defective 2342-ras cells (○) were treated with differentiation-inducing medium supplemented with concentrations of TPA ranging from 1 nM to 1 μM. The PKC activity in the cell extracts prepared from each culture was measured as described in Materials and Methods. Each value represents the average of four independent determinations, with the exception of the 500 nM 2342-ras group which was assayed twice. The high and low values for each group of determinations are indicated by the range bars.

Protein Kinase C Activity (pmol/min/mg protein)

CONCENTRATION OF TPA (nM)

Figure 4. Western blot analysis of PKC protein in TPA-treated 2342 and 2342-ras cell extracts. Cultures were induced to differentiate in medium containing 0 nM, 100 nM, or 1 μM TPA. Equal amounts of total protein from each extract were treated with differentiation-inducing medium supplemented with concentrations of TPA ranging from 1 nM to 1 μM. After 48 h, RNA and protein were isolated from the cultures and examined for expression of several muscle-specific markers, including muscle creatine kinase (MCK), troponin I (TnI), and the muscle regulatory factor, MyoDl. To examine the effect of TPA on cell fusion, parallel cultures were treated with TPA-supplemented F12 differentiation-inducing medium and immunohistochemically stained with the monoclonal antibody, MF-20 (3), to detect skeletal myosin heavy chain proteins.

Treatment of 2342 cells with TPA had only a minor effect on muscle-specific gene expression. All experimental groups possessed high levels of MCK activity and had a high percentage of cells staining positively for skeletal myosin heavy chains (Table I). Northern blot analysis of the RNA isolated from each experimental group demonstrated that the TPA-treated cultures expressed both TnI and MyoDl mRNAs (Fig. 5). These results show that reducing PKC activity does not significantly affect the molecular mechanisms through which muscle-specific gene expression is activated or maintained in 2342 cells.

The 2342 cultures that were stained immunohistochemically for myosin heavy chain protein also were used to assess whether TPA-treated cells formed multinucleate myofibers as efficiently as untreated control cells. As shown in Table I and in Fig. 6, exposing 2342 cells to 100 nM, 500 nM, or 1 μM TPA decreased the number of myosin-positive multinucleate cells in the differentiated cultures by up to 80%. Interestingly, the inhibition of myoblast fusion was apparent only after treatment of the cultures with concentrations of TPA that resulted in decreased PKC activity and a loss of immunoreactive PKC protein (Figs. 3 and 4), suggesting that a critical level of PKC activity is essential for optimal myocyte fusion, but not for maintaining the transcriptional activity of the muscle-specific gene set (Table I and Fig. 5).

Since TPA is a lipophilic compound (reviewed in reference 32), it is possible that the levels of TPA that were used in these experiments inhibited cell fusion by altering the struc-
Table I. Effects of TPA on the Differentiation of 23A2 Myoblasts

| Concentration of TPA (nM) | MCK activity* | Percent myosin positive cells† | Percent myofibers§ |
|--------------------------|---------------|-------------------------------|------------------|
| 0                        | 3,020         | 83                            | 76               |
| 1                        | 3,205         | 85                            | 77               |
| 10                       | 2,345         | 75                            | 68               |
| 100                      | 1,505         | 76                            | 47               |
| 500                      | 2,075         | 67                            | 16               |
| 1,000                    | 1,865         | 67                            | 16               |

* Protein extracts isolated from differentiation-induced cultures were assayed for the muscle-specific isoform of creatine kinase (MCK) as described in Materials and Methods. The values represent the averages of two independent experiments and are presented as creatine kinase enzyme units per gram of total protein. Each value has been adjusted for the MCK activity measured in 23A2 myoblasts which was 105 enzyme units per gram of protein.
† 23A2 cells were induced to differentiate in the presence of increasing concentrations of TPA and then stained immunochemically for skeletal myosin heavy chain protein as described in Materials and Methods. The percentage of cells expressing the myosin heavy chain protein was calculated by dividing the number of nuclei in myosin-positive cells by the total number of nuclei counted. Five randomly chosen microscope fields (>100 nuclei per field) were analyzed for each experimental group, and the results from two independent experiments were averaged to obtain the values presented.
§ Cultures used for the determination of percent myosin positive cells also were used for the determination of percent myofibers by dividing the number of nuclei contained in cells possessing >32 nuclei by the total number of nuclei counted per field. Five randomly chosen microscope fields (>100 nuclei per field) were examined for each experimental group, and the results from two independent experiments were averaged to obtain the values presented.

Figure 5. Northern blot analysis of total RNA isolated from TPA-treated 23A2 cells. Cultures were induced to differentiate in medium supplemented with concentrations of TPA from 1 nM to 1 μM. 15 μg of RNA from each culture were electrophoresed through an agarose/formaldehyde gel, transferred to nitrocellulose, and probed for MyoD1 and Tn1 mRNAs as described in Materials and Methods. Control lanes contain 15 μg of RNA isolated from untreated 23A2 myoblasts (23A2 B) and myofibers (23A2 F).

TPA Treatment Does Not Reverse the Differentiation-defective Phenotype of 23A2 ras Cells

Our examination of the role of PKC activity in the differentiation of wild-type 23A2 myoblasts suggests that a reduction in PKC activity certainly is not deleterious to 23A2 cells and that a modest reduction may be essential for the proper execution of the skeletal muscle differentiation program. Therefore, since differentiation-defective 23A2 ras cells possess elevated levels of PKC activity, we decided to test whether myogenesis could be restored in these cultures by lowering the levels of PKC. 23A2 pT249a2 cells were treated with F12 differentiation-inducing medium supplemented with concentrations of TPA from 1 nM to 1 μM. After 4 d, the cultures were fixed, stained immunochemically with the MF-20 monoclonal antibody, and observed for morphological differentiation. In all cases, neither myofiber formation nor myosin heavy chain gene expression was detected (data not shown). RNA was isolated from parallel cultures and Northern hybridizations performed to detect expression of muscle-specific genes. As expected from the absence of morphological differentiation, the TPA-treated ras cells did not express Tnl or MyoD1 mRNAs, but did continue to express significant levels of mRNA transcribed from the transfected H-ras gene (Fig. 7 A). We conclude from these studies that continued high levels of PKC activity are not required to maintain the differentiation-defective phenotype of this established ras cell line.

We also examined whether TPA treatment affected the level of ras protein in the cells. 23A2 and 23A2 pT249a2 cultures were treated for 48 h with ITS medium containing 500 nM TPA and total ras protein was immunoprecipitated from 35S-labeled cell extracts using the pan-reactive ras monoclonal antibody, Y13-259 (20). The results of this analysis demonstrate that both 23A2 and 23A2 ras cells exhibit a measurable decrease in ras protein after treatment with TPA (Fig. 7 B). However, as was indicated by the Northern analysis (Fig. 7 A), TPA-treated ras cells continue to express activated ras p21 and possess at least twofold more ras protein than TPA-treated wild-type cells (Fig. 7 B). Since the 23A2 ras cultures do not differentiate in the presence of TPA, either the amount of activated ras p21 remaining in the ras cells is sufficient to inhibit differentiation or, alternatively, high levels of ras p21 (like high levels of PKC activity) are not required to maintain the differentiation-defective phenotype of the 23A2 pT249a2 cells.

Initiation of the Differentiation-defective Phenotype of 23A2 ras Cells Requires PKC

Although it appears that an elevated level of PKC activity is not required to maintain the nonmyogenic phenotype of 23A2 ras cells, activated ras p21 may rely on PKC to initiate its negative effects on skeletal myogenesis. To examine this possibility stable transfection experiments were performed.
Wild-type 23A2 myoblasts were maintained in normal growth medium or in growth medium supplemented with 500 nM TPA. TPA-treated and untreated 23A2 myoblasts were cotransfected with a single calcium phosphate precipitate containing either a selectable marker (pKOneo) and the oncogenic form of the human H-ras gene (pT24) or pKOneo and the human H-ras proto-oncogene (p3B). Each transfected culture then was divided into experimental groups that were maintained in selective medium supplemented with TPA for various lengths of time (Fig. 8). After colonies had formed (~15 d), the cultures were treated with F12 differentiation-inducing medium (supplemented with TPA where indicated), fixed, stained with Giemsa, and analyzed for the percentage of myogenic colonies (Fig. 8). For the control groups transfected with the H-ras proto-oncogene (groups A and B), 86% of the untreated colonies and 75% of the TPA-treated colonies were differentiation competent. We attribute the modest decrease in myogenesis observed for group B to the difficulty of scoring myogenesis in cultures where myocyte fusion is reduced by TPA (Fig. 6). As shown in Fig. 8, a two-, three-, and fivefold increase in the percentage of differentiated colonies was obtained when the cells were treated with TPA on days 10, 4, and -1, respectively (compare Group C with Groups D, E, and F), demonstrating that early treatment with TPA enhances the frequency of myogenic colonies. Similarly, the longer TPA remained on the cells after transfection, the less effective ras p21 was in inhibiting myogenesis. Cultures maintained in TPA for 4, 10, or 19 d produced 16, 35, and 54% differentiated colonies, respectively (compare Groups H, G, and F).

The results presented in Fig. 8 were compiled from three independent transfection experiments. For Experiment 3, RNA was isolated from each group and Northern blot hybridizations performed to assess the levels of MyoD1, TnI, and ras mRNAs in each culture. As expected, the levels of MyoD1 and TnI gene expression determined for each group reflected the percentage of colonies that differentiated into skeletal myofibers (Fig. 9). As previously described for the 23A2 pT249a2 cell line, all groups treated with TPA exhibited reduced levels of activated ras gene expression (Fig. 9) and it is possible that this contributes to the increase in myogenic competence that is observed. On the other hand, it is not known how much activated ras p21 is required to inhibit 23A2 myogenesis, although the high frequency of non-myogenic colonies arising from a neo/ras cotransfection (Group C) suggests that colonies expressing even minimal levels of ras are scored as non-myogenic by our assay. Therefore, we conclude that ras p21 relies on PKC activity to initiate the intracellular changes responsible for inhibiting myogenesis. However, based on our results with the 23A2 pT249a2 cell line (Fig. 6), a continued high level of PKC activity is not required to maintain the differentiation-defective phenotype of ras myoblasts.
Discussion

In this study, we have examined the changes in protein kinase C activity that are associated with the terminal differentiation of 23A2 myoblasts and the differentiation-defective phenotype of 23A2 ras cells. Our interest in PKC as a major component of the signal transduction pathway(s) that regulates skeletal myogenesis comes from two lines of evidence. First, experiments with primary avian myoblasts have demonstrated that exogenous activators of PKC, such as phorbol ester tumor promoters, adversely affect several developmental events associated with skeletal myogenesis, including the fusion of cells and the expression of muscle-specific genes (8, 41). Second, several laboratories have found that expression of activated ras p21 in mammalian cells leads to a constitutive increase in intracellular diacylglycerol (18, 24, 28, 39, 50) which serves as an endogenous activator of PKC (reviewed in references 34, 35). These observations support the hypothesis that PKC plays an important role in controlling normal skeletal myogenesis and that changes in PKC activity may be instrumental in the ras-induced inhibition of skeletal muscle differentiation.

The differentiation of wild-type 23A2 myoblasts is associated with a 50% reduction in PKC activity, suggesting that a decrease in PKC levels may be critical to achieving and maintaining a terminally differentiated phenotype. Prolonged
treatment of differentiation-induced 23A2 cells with TPA results in a further reduction of PKC activity and although this treatment has no effect on biochemical differentiation, the fusion of the cells inhibited. This result indicates that a certain level of PKC activity is required for proper morphological differentiation. In this regard, David et al. (10) have examined the biochemical events associated with the differentiation of primary chick myoblasts and have shown that PKC

![Figure 8. TPA treatment of 23A2 cells blocks the inhibition of myogenesis by activated ras p21. 23A2 cells were treated with 500 nM TPA for various times before or after transfection with pKOneo and either the H-ras proto-oncogene (Control) or the H-ras oncogene (ras). After selection of G418-resistant colonies, the cultures were induced to differentiate and then analyzed for the number of myogenic colonies as described in Materials and Methods. The results of the colony counts for three independent transfection experiments are presented individually (Exp. 1, Exp. 2, and Exp. 3) and as an average for each experimental group. (a) The number of myogenic colonies expressed as a percentage of the total number of colonies analyzed. (b) The average of the percentages obtained from Exps. 1, 2, and 3.](image)

![Figure 9. Northern blot analysis of total RNA isolated from Groups A-H of Exp. 3 in Fig. 8. 15 μg of RNA from each experimental group were electrophoresed through an agarose/formaldehyde gel, transferred to nitrocellulose, and probed for MyoD1 and TnI mRNAs as described in Materials and Methods. The filter then was stripped as described in Fig. 7 and reprobed for expression of the transfected H-ras oncogene (ras). Control lanes contain 15 μg of total RNA from 23A2 myofibers (23A2 MF), differentiation-induced 23A2 pI249a2 cells treated (23A2-ras + TPA) or not treated (23A2-ras) with 500 nM TPA, and C3H10T1/2 fibroblasts (C3H10T1/2). The lower panel shows the ethidium bromide-stained gel before transfer of the RNA to nitrocellulose.](image)
activity stimulates membrane events, such as the influx of extracellular calcium, that are necessary prerequisites for cell fusion.

Differentiation-defective 23A2 ras cells possess elevated levels of PKC activity as judged by three separate measurements: in vitro kinase assays (Fig. 1), TRE-CAT assays (Fig. 2), and Western analyses (Fig. 4). Prolonged treatment of 23A2 pT249a2 cells with TPA effectively reduced PKC activity to undetectable levels, but did not restore myogenic competence to the cultures (Fig. 7 A). In contrast, when 23A2 cells were pretreated with TPA and then transfected with the ras oncogene, normal biochemical differentiation was achieved in a high percentage of the transfected colonies (Figs. 8 and 9, Group F). This observation, combined with our analysis of the 23A2 pT249a2 cell line, suggests that ras relies on PKC to initiate the inhibition of myogenesis, but that a continued high level of PKC expression is not required for the block in myogenesis to be maintained.

Examination of the levels of ras protein in wild-type and ras-transfected 23A2 cells reveals a decrease in ras p21 after treatment with TPA (Fig. 7 B). The decrease observed is similar for both cell types and the 23A2 pT249a2 retain at least twofold more ras p21 than the wild-type cells. Since the Y13-259 ras antibody used for this experiment does not distinguish between the endogenous mouse and transfected human ras p21 species, it is impossible to assess the level of activated ras p21 in the TPA-treated 23A2 pT249a2 cells. However, it is apparent from the Northern analyses that significant levels of the activated human H-ras mRNA persist in ras-transfected cells that are exposed to TPA (Figs. 7 A and 9). The contribution of a modest reduction in ras p21 expression to the results reported here is not clear. The inability of TPA treatment to restore differentiation in 23A2 pT249a2 cells would suggest that the level of activated ras p21 remaining in these cells is sufficient to inhibit differentiation or, alternatively, that ras p21 (like PKC activity) is not required to maintain the non-myogenic phenotype of 23A2 ras cells. We favor the former possibility since Gossett et al. (21) have demonstrated that sustained expression of an inducible H-ras oncogene is absolutely essential for the maintenance of the ras-induced inhibition of C2 skeletal myogenesis. Similarly, the partial suppression of ras p21 expression by TPA cannot explain the results of the transfection experiments reported in Fig. 8 for the following reasons. First, our experience with 23A2 cells has demonstrated that the efficiency of cotransfection in these cultures is, at best, 80–90% (Vaidya, T., and E. Taparovsky, unpublished results). Therefore, it is likely that the 11% differentiation-competent colonies observed for Group C (Fig. 8) represent cells that are expressing only the neomycin resistance gene and the remaining differentiation-defective colonies (89%) represent cells that have been cotransfected with neo and ras and, thus, express various amounts of activated ras protein. Second, Northern analysis of RNA isolated from pooled populations indicates that the level of activated H-ras mRNA is decreased similarly in all TPA-treated groups (compare Fig. 9, Groups D–H), yet the percentage of myogenic colonies varies from 23% in Group D to 57% in Group F. These observations support the conclusion that the activated ras protein relies on PKC activity to initiate its full negative effects on skeletal muscle differentiation. In this regard, studies exploring the mitogenic effects of ras p21 in mammalian fibroblasts have demonstrated that the metabolic consequences of ras activation can be separated into PKC-dependent and PKC-independent events (6, 31). Our results suggest that the effects of activated ras p21 on 23A2 myoblasts show a similar pattern with short-term PKC-dependent and long-term PKC-independent events collaborating to inhibit skeletal muscle differentiation.

In mammalian fibroblasts, activated ras gene expression leads to increased diacylglycerol production, which results in the activation and subsequent downregulation of PKC (24, 50). In previous studies, we have demonstrated that ras-transfected C3H10T1/2 cells, the fibroblast cell line from which the 23A2 myogenic cell line was derived originally (26), show a 40% downregulation of PKC activity and protein (49). Although we have preliminary data indicating that 23A2 ras cells possess elevated levels of diacylglycerol compared to control cells (Vaidya, T., and E. Taparovsky, unpublished results), our present studies have shown that 23A2 ras cells possess at least 10 times more PKC protein (Fig. 4) and two- to threefold more PKC activity (Figs. 1 and 3) than wild-type myofibers. Why PKC accumulates to a high level in 23A2 ras cells is not clear. One possibility is that ras induces expression of additional isozymes of PKC that display partial or complete resistance to downregulation. Cooper et al. (9) have demonstrated that BC3H1 myocytes express a type II PKC isozyme that displays a preference for phosphorylating vinculin, not histone, and is refractile to downregulation by TPA. We have addressed whether a similar isozyme switch has occurred in 23A2 ras cells using in vitro kinase assays and Western blot analyses with a polyclonal antiserum raised against all of the major PKC isozymes to measure how the PKC pool in 23A2 ras cells responds to treatment with TPA. Our results indicate that the pool of PKC produced by the 23A2 ras cells and the pool produced by wild-type 23A2 cells are equally responsive to phorbol ester downregulation (Figs. 3 and 4). This suggests that 23A2 ras cells accumulate PKC because of an increased rate of PKC synthesis, and in vivo labeling experiments currently are being performed to address this possibility. In addition, we are intrigued by the observation that the 23A2 ras cells contain substantially more PKC protein than predicted from the kinase activity values (compare Figs. 3 and 4). In preliminary studies, we have found that the partial purification of PKC from 23A2 ras cell extracts dramatically increases the kinase activity of the protein (Weyman, C., and C. Ashendel, unpublished results). This suggests that 23A2 ras cells may produce an unknown inhibitor of PKC activity. Whether this inhibitor influences directly the accumulation of PKC in the ras cells remains to be explored.

We have used the 23A2 myogenic cell line to address fundamental questions concerning cellular growth control. Defining the molecular mechanisms through which an activated ras oncogene transforms cells and subverts the proper control of cellular differentiation is an intriguing problem. Our studies demonstrate that the signal transduction pathways used by ras p21 to inhibit 23A2 skeletal myogenesis rely on PKC activity and have as a nuclear target the down-regulation of the MyoD1 regulatory gene. Interestingly, growth factor–mediated signal transduction remains the catalyst in initiating myogenic differentiation, since wild-type cells that express high levels of MyoD1, or ras cells engineered to express a MyoD1 cDNA, do not differentiate in
the presence of serum, FGF, or TGF-β (26, 30, 46). In addition, our studies indicate that the growth factors important to skeletal myogenesis may operate independently of PKC activity, since severely reducing PKC levels in proliferating 23A2 myoblasts does not elicit a precocious differentiation response. Undoubtedly, an important question for future studies will be to address how the myogenic regulatory factors, such as MyoD1, are modified in cells exposed to these various agents, since it is apparent that subtle changes in the state of these proteins are essential to the proper initiation and maintenance of the myogenic lineage.

We thank our colleagues in the laboratory for helpful discussion, Connie Philbrook and Connie Reynolds for excellent secretarial assistance, and Dr. Stephen Konieczny for critically reading the manuscript.

T. B. Vaidya and C. M. Weyman are David Ross Fellows of Purdue University. This work was supported by grants awarded to E. J. Taparowsky from the National Science Foundation (DCB-8903515) and to C. L. Ashendel from the American Cancer Society (BC-636).

Received for publication 4 October 1990 and in revised form 12 March 1991.

References

1. Alemd, S., and F. Tat6. 1987. Interaction of retroviral oncogenes with the differentiation program of myogenic cells. Adv. Cancer Res. 49:1-28.

2. Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, J. H. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin. 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated transacting factor. Cell. 49:729-739.

3. Barker, A. J. 1989. The ras gene family: From genetics to molecular mechanisms. Annu. Rev. Biochem. 58:779-827.

4. Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. (Eur. Mol. Biol. Organ.) 8:790-799.

5. Cai, H., J. Szczepanski, and G. M. Cooper. 1990. Effects of a dominant inhibitory Ha-ras mutation on mitogenic signal transduction in NIH3T3 cells. J. Cell Biol. 95:763-770.

6. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

7. Cohen, R., M. Pacifi, N. Rabinstein, J. Biehl, and H. Hottzter. 1977. Effect of a tumour promoter on myogenesis. Nature (Lond.) 266:538-540.

8. Cooper, D. R., J. E. Watson, M. Acevedo-Duncan, R. J. Pellet, M. L. Standerfer, and R. V. Fares. 1989. Retention of specific protein kinase C isozymes following chronic phorbol ester treatment in BCh3-1 myocytes. Biochem. Biophys. Res. Commun. 161:327-334.

9. Davis, J. D., C. R. Faser, and G. P. Perrot. 1990. Role of protein kinase C in chick embryo skeletal myoblast fusion. Dev. Biol. 139:89-99.

10. Davis, R. L., H. Weintraub, and A. B. Lassar. 1989. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell. 51:987-1000.

11. Dotto, G. P., L. F. Parada, and R. A. Weinberg. 1985. Specific growth response of ras-transformed embryoblast fibroblasts to tumour promoters. Nature (Lond.) 316:472-475.

12. Emerson, C. P., Jr., D. A. Fischman, B. Nadal-Ginard, and M. A. Q. Siddiqui. 1986. Molecular Biology of Muscle Development. Alan R. Liss, Inc., New York.

13. Enkemann, S. A., S. F. Konieczny, and E. J. Taparowsky. 1990. Adenvirus 5 E1A represses muscle-specific enhancers and inhibits expression of the myogenic regulatory factor genes, MyoD1 and myogenin. Cell Growth Differ. 1:375-382.

14. Fusaro, G., E. Taparowsky, J. Fiddes, M. Wigler, and M. Goldfarb. 1983. Sequence and structure of the coding region of the human H-ras-1 gene from T24 bladder carcinoma cells. J. Mol. Appl. Genet. 2:173-180.

15. Fleischman, L. F., S. B. Chahwala, and L. Cantley. 1986. Ras-transformed cells: altered levels of phosphatidylinostol-4,5-bisphosphate and catabolite gene activator protein. Science (Wash., D.C.) 231:407-411.

16. Finlin, J. R., and K. A. Magri. 1989. Effects of growth factors on myo-
scription of the myogenic regulatory gene MyoD. Mol. Cell. Biol. 9:3576–3579.

47. Weinstein, I. B. 1981. Current concepts and controversies in chemical carcinogenesis. J. Supramol. Struct. Cell. Biochem. 17:99–120.

48. Weinstein, I. B. 1987. Growth factors, oncogenes, and multistage carcinogenesis. J. Cell. Biochem. 33:213–224.

49. Weyman, C. M., E. J. Taparonow, M. Wolfson, and C. L. Ashendel. 1988. Partial down-regulation of protein kinase C in C3H10T1/2 mouse fibroblasts transfected with the human Ha-ras oncogene. Cancer Res. 48:6535–6541.

50. Wolfman, A., and I. G. Macara. 1987. Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in ras-transformed fibroblasts. Nature (Lond.). 325:359–361.

51. Wright, W. E., D. A. Sassoon, and V. K. Lin. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell. 56:607–617.

52. Yutzey, K. E., R. I. Kline, and S. F. Konieszny. 1989. An internal regulatory element controls troponin I gene expression. Mol. Cell. Biol. 9:1397–1405.