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

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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 nonmyogenic 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 differentiation of skeletal muscle cells involves the withdrawal of proliferating myoblasts from the cell cycle, the formation of multinucleate myofibers and the transcriptional activation of a complete set of muscle-specific genes (13). Experiments with cultured myogenic cell lines have led to the identification of multiple genetic and environmental factors that influence the establishment and the proper differentiation of the myogenic lineage. The protein products of at least five genes, *MyoD1*, *myd*, *myogenin*, *Myf-5*, and *MRF4*, are important to the regulation of myogenic differentiation (5, 11, 38, 40, 51). In addition, depletion of serum growth factors from the culture medium is essential for myocyte fusion and for the coordinate expression of the contractile protein gene set (reviewed in reference 19), suggesting that growth factor signal transduction pathways control these developmental events. In this regard, several laboratories have shown that oncogenes such as *ras*, *fos*, and *src* inhibit skeletal muscle differentiation, presumably by interfering with the proper functioning of intracellular signalling pathways (see reference 1 for review; 26, 30, 36, 37).

The signal transduction pathways used by the various inducers and inhibitors of skeletal myogenesis remain unknown. Recently, our laboratory has shown that the differentiation of the mouse myogenic cell line, 23A2, is blocked after transfection of the cells with the activated form of the human H-*ras* gene (26) or by exposure of the cells to purified fibroblast growth factor (bFGF) or transforming growth factor β1 (TGF-β) (46). In each case, the inhibition of myogenesis is associated with the downregulation of the myogenic regulatory gene, *MyoD1* (26, 46). Interestingly, transfection of 23A2 cells with a constitutive *MyoD1* cDNA expression vector restores myogenesis in the *ras*-inhibited cultures, but does not reverse the inhibition induced by growth factors (26, 46). This suggests that *ras*, bFGF, and TGF-β similarly affect an intracellular pathway involved in regulating *MyoD1* gene expression, but that the growth factors affect an additional pathway, perhaps involved in the posttranslational modification of the *MyoD1* protein, that is equally critical for the proper execution of skeletal myogenesis.

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 *MyoDl* 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 *MyoDl* 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 fusion, 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 *MyoDl* 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 *MyoDl* 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 μg/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 pT249a2 cell line is a subclone of 23A2 pT249 (26) and is transfected stably with the oncogenic form of the human H-*ras* gene. For all experiments, the culture conditions for the 23A2 pT249a2 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, 5×10^5 cells were seeded per 100-mm dish. On the next day precipitates containing 30 ng of pKOneo (43) and 300 ng of either p3B (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 μg/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 discrete myofibers over the total number of colonies examined. For experimental groups treated with TPA or the nonfunctional TPA analogue 4α-phorbol 12-myristate 13-acetate (4α-PMA) (L. C. Services Corporation, Woburn, MA), a 2 mg/ml solution in ethanol (100%) was used to supplement the growth or differentiation medium. For transient transfections, 1×10^5 23A2 myoblasts or 23A2 pT249a2 cells were seeded per 100-mm dish. After 2 d, precipitates containing 5 μg of 5× TRE CAT (2) and 5 μg 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 myofiber transfections were performed exactly as described above with the exception that the cells were seeded at 4×10^5 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 β-galactosidase gene (RSVlacZ) as described in Nielson 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 immunochemically 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 μg 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, 2 mM EDTA and 0.5% SDS containing 100 μg/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 *MyoDl*-specific fragment from pEMC11s (11), were labeled, using α-^{32}P dCTP (sp act 3,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) and the oligolabeling procedure

1. *Abbreviations used in this paper*: CAT, chloramphenicol acetyltransferase; 4α-PMA, 4α-phorbol 12-myristate 13-acetate; MCK, muscle creatine kinase; PKC, protein kinase C; TnI, troponin I; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

of Feinberg and Vogelstein (16, 17). Denatured probes (sp act > 1×10^8 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-cm^2 flask and extracts were prepared after 48 h. To obtain myofiber extracts, cultures were seeded in growth medium at 4×10^5 cells per 75-cm^2 flask on day 0, treated with ITS medium on day 2, and extracts isolated on day 4. Cultures were washed twice with cold (4°C) PBS (145 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 EGTA, containing 500 μg/ml digitonin, 10 μg/ml of 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, Div., 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 $MgCl_2$, 0.5 mM $CaCl_2$, 10 mM *p*-nitrophenyl phosphate, 20 μM γ^{32}P ATP (sp act 0.2–1.0 Ci/mmol) containing 80 μg/ml phosphatidylserine and 240 μg/ml lysine-rich histone H1 (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 EGTA and 15 mg/ml CHAPS, in the absence of $CaCl_2$ 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 ^{32}P incorporated into histone determined by scintillation counting. PKC activity is calculated as picomoles of ^{32}P 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% 2-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 ^{125}I-protein A (sp act 10–100 mCi/mg; New England Nuclear, Boston, MA) per 100 ml of incubation buffer for a minimum of 2 h. After a final set of washes with 1% Tween 20 in TNS the filters were exposed to XAR film (Kodak) at −80°C with an intensifying screen.

Immunoprecipitations

Cells were plated in normal growth medium at a density of 2×10^5 cells per 60-mm dish and on the next day, fed fresh medium with or without 500 nM TPA. 24 h later, the cultures were fed methionine-free DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 20 mM Hepes, 5% fetal bovine serum, P/S, and 500 nM TPA (where appropriate) for 2 h, and methionine-free DMEM containing 200 μCi/ml of Trans^{35}S (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. 4×10^7 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 μl 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 En^{3}hance (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 PSMF. 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, *MyoD1*, since introducing a constitutively expressed *MyoD1* 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 *MyoD1* 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 myofiber 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

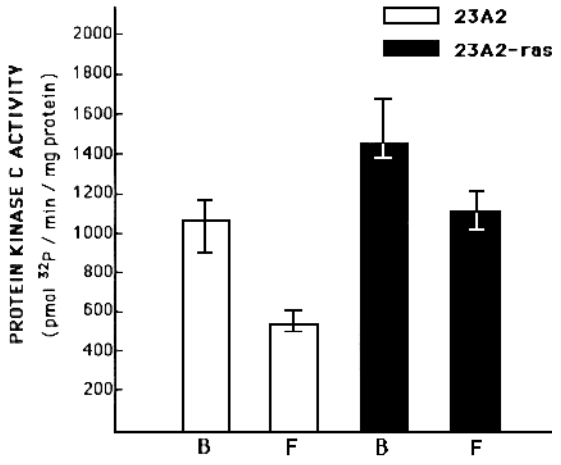


Figure 1. PKC activity in wild-type 23A2 cells (*23A2*) and in differentiation-defective 23A2 pT249a2 cells (*23A2-ras*). Extracts from proliferating (*B*) and differentiation-induced (*F*) cultures were assayed for the ability to phosphorylate histone H1 in a calcium- and phospholipid-dependent manner as described in Materials and Methods. PKC activity is expressed as picomoles of ^{32}P incorporated into histone per minute per milligram of protein. Each value represents the average of six independent determinations of PKC activity. The high and low values for each group of determinations are indicated by the range bars.

myofibers. This higher level was approximately equal to that of proliferating 23A2 myoblasts, suggesting that PKC activity may be involved in maintaining the undifferentiated state of the *ras* cultures. The increased level of PKC activity associated with *ras* expression is not unique to the 23A2 pT249a2 cell line since in vitro kinase assays performed on two additional 23A2 *ras* clones, 23A2 pT248 and 23A2 pT2410 (26), produced similar results (data not shown).

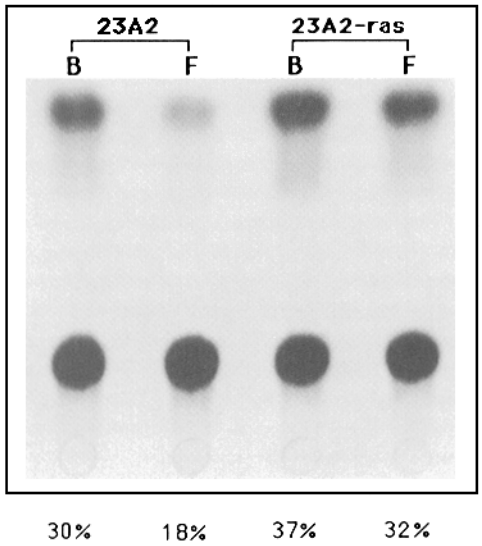


Figure 2. TRE-CAT activity in 23A2 and 23A2-*ras* cells. Cells were transfected transiently with 5 μg of the 5XTRE-CAT reporter plasmid and 5 μg of the RSVlacZ reference plasmid as described in Materials and Methods. Extracts were prepared from proliferating (*B*) and differentiation-induced (*F*) cells, normalized for β-galactosidase activity, and then assayed for CAT activity as described in Materials and Methods. CAT activity is reported as the percent conversion of chloramphenicol to its acetylated form. The CAT assay shown is one of two independent experiments whose results were averaged to obtain the percent conversions presented.

To confirm that the levels of PKC activity measured in vitro reflect the activity of PKC in vivo, 23A2 and 23A2 pT249a2 cells were assayed for their ability to express a chloramphenicol acetyltransferase (CAT) reporter gene controlled by a PKC responsive DNA element (TRE) (2). 23A2 and 23A2 pT249a2 cells were transfected transiently with the TRE-CAT reporter gene and proliferating and differentiation-induced cultures monitored for CAT activity (see Materials and Methods for details). As shown in Fig. 2, treatment of 23A2 myoblasts with ITS resulted in a 40% decrease in TRE-CAT activity, which paralleled the decrease in the in vitro kinase activity measured in these same cultures (Fig. 1). As expected, 23A2 pT249a2 cells also exhibited a modest decrease in TRE-CAT activity when treated with ITS. However, CAT expression remained approximately twofold higher in the differentiation-defective *ras* cultures than in the differentiation-competent 23A2 myofiber cultures. These studies agree with the previous in vitro kinase assay results (Fig. 1) and suggest that the increased levels of PKC activity found in 23A2 *ras* cells may be important to their differentiation-defective phenotype.

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-

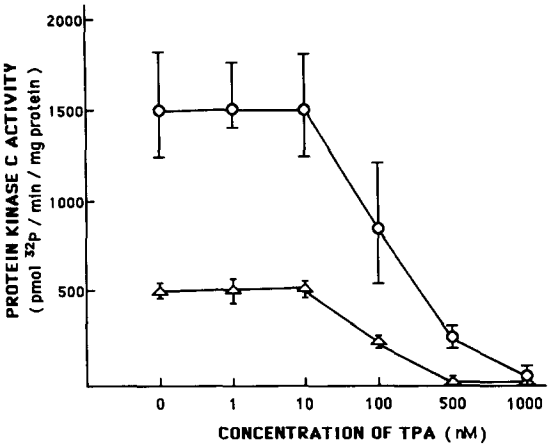


Figure 3. PKC activity in TPA-treated 23A2 and 23A2-*ras* cells. 23A2 cells (△) and the differentiation-defective 23A2-*ras* cells (○) were treated with differentiation-inducing medium supplemented with concentrations of TPA ranging from 1 nM to 1 μM. The PKC activity in the cell extracts prepared from 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 23A2-*ras* group which was assayed twice. The high and low values for each group of determinations are indicated by the range bars.

onstrate that the pool of PKC protein present in the *ras* and the wild-type cells is equally responsive to downregulation by TPA.

To confirm that the decrease in PKC activity observed after TPA treatment resulted from proteolysis of the enzyme, cell extracts were isolated from 23A2 and 23A2 *ras* cells that had been treated for 48 h with ITS medium containing 0 nM, 100 nM, or 1 μM TPA. Equal amounts of total protein from each extract were electrophoresed through an SDS–polyacrylamide gel, blotted to nitrocellulose, and then reacted with a chicken polyclonal antiserum prepared against total rat brain PKC (49). As shown in Fig. 4, treatment of the cells with concentrations of TPA that produced a decrease in PKC

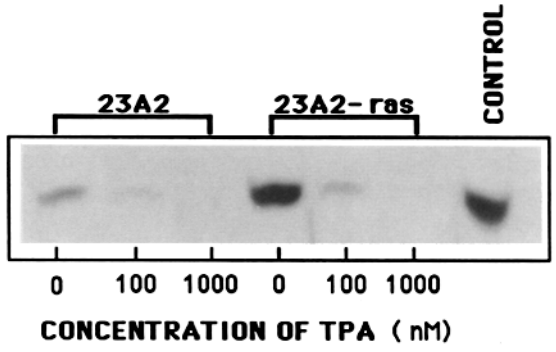


Figure 4. Western blot analysis of PKC protein in TPA-treated 23A2 and 23A2-*ras* cell extracts. Cultures were induced to differentiate in medium containing 0 nM, 100 nM, or 1 μM TPA. Cell extracts were prepared and 66 mg of total protein from each extract were resolved by SDS-PAGE, transferred to nitrocellulose, and hybridized with anti-rat brain PKC egg yolk antibodies as described in Materials and Methods. The control lane contains ~125 ng of purified rat brain PKC.

activity (Fig. 3) resulted in a corresponding loss of immunoreactive protein. 23A2 and 23A2 *ras* cultures treated with 1 μM TPA contained undetectable levels of PKC. Therefore, based on TPA responsiveness, we conclude that the PKC protein pool produced by the 23A2 pT249a2 cells is indistinguishable from the PKC pool found in the wild-type 23A2 cell line. In addition, these studies provide preliminary support for the hypothesis that the increase in PKC protein and activity observed in 23A2 *ras* cells is due to alterations in the intracellular pathways regulating PKC synthesis, and not to changes in the pathway responsible for PKC proteolysis.

Downregulating PKC Affects the Morphological Differentiation of 23A2 Cells, But Not the Transcriptional Activation of Muscle-specific Genes

Since we observed a 50% decrease in PKC activity after the treatment of 23A2 myoblasts with differentiation-inducing medium, we used the ability of TPA to downregulate PKC in these cells to address whether the level of PKC remaining in 23A2 myofibers is essential for the full expression of a muscle phenotype. 23A2 cultures were treated with ITS medium supplemented with concentrations of TPA 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, *MyoDI*. To examine the effect of TPA on cell fusion, parallel cultures were treated with TPA-supplemented F12 differentiation-inducing medium and immunochemically stained with the monoclonal antibody, MF-20 (3), to detect skeletal myosin heavy chain proteins.

Treatment of 23A2 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 *MyoDI* 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 23A2 cells.

The 23A2 cultures that were stained immunochemically 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 23A2 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	Myogenic marker		
	MCK activity*	Percent myosin positive cells‡	Percent myofibers§
nM			
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 ≥2 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.

ture of the myocyte plasma membrane. To examine this further, 23A2 myoblasts were treated with F12 differentiation-inducing medium containing 1 μM of the TPA analogue 4α-PMA. Although 4α-PMA has similar lipophilic properties as TPA, it does not trigger the activation or downregulation of PKC (reviewed in reference 32). In this control experiment, no decrease in cell fusion was detected (Fig. 6), demonstrating that the ability of TPA to inhibit myocyte fusion is linked directly to its ability to alter PKC activity in these cells.

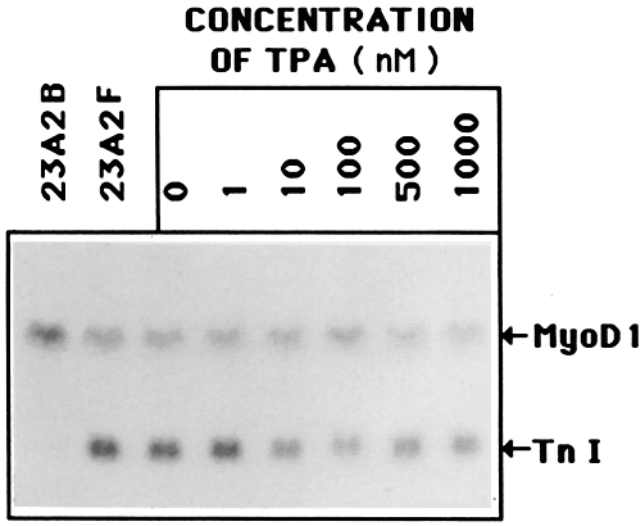


Figure 5. Northern blot analysis of total RNA isolated from TPA-treated 23A2 cells. Cultures were induced to differentiate in medium supplemented with concentrations of TPA from 1 nM to 1 μM. 15 μg of RNA from each culture were electrophoresed through an agarose/formaldehyde gel, transferred to nitrocellulose, and probed for *MyoD1* and TnI 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 TnI 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 ^{35}S-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.

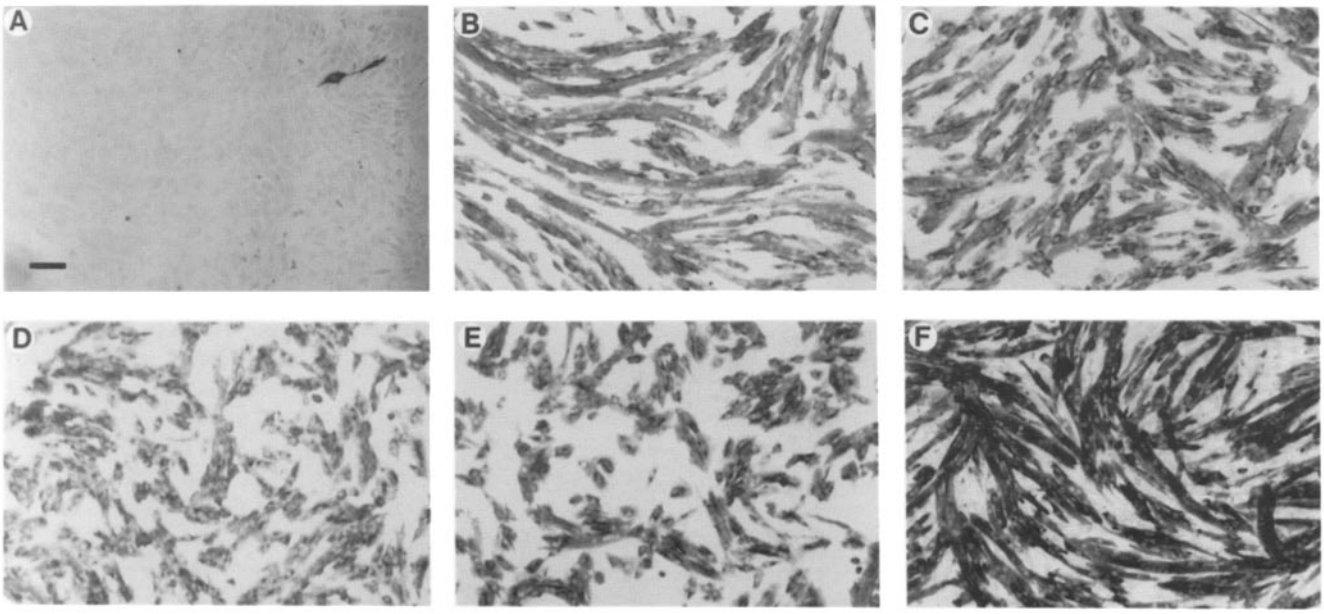


Figure 6. Inhibition of myoblast fusion by TPA. 23A2 myoblasts were induced to differentiate in medium supplemented with concentrations of TPA from 1 nM to 1 μM. The differentiated cultures were fixed, immunochemically stained to detect myosin heavy chain protein (see Materials and Methods for details), and photographed under bright light conditions. *A* shows the level of background staining obtained with undifferentiated 23A2 myoblast cultures. *B–E* show the morphology of 23A2 myofibers that have differentiated in the presence of 0 nM (*B*), 10 nM (*C*), 100 nM (*D*), and 1 μM TPA (*E*). *F* shows 23A2 cells that were induced to differentiate in the presence of 1 μM 4α-PMA, a non-functional analogue of TPA. Bar, 100 μm.

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). For the parallel experimental groups transfected with the H-*ras* oncogene (Groups C and F), only 11% of the colonies in the untreated cultures were competent for differentiation, while 54% of the colonies arising from the TPA-treated cultures contained differentiated cells. The fivefold increase in the myogenic competence of *ras*-transfected cells that were pretreated with TPA suggests that the downregulation of PKC by TPA prevents activated *ras* p21 from exerting its full negative effects on skeletal myogenesis.

We next compared the results of Groups C and F with the results obtained from additional experimental groups in which TPA was added or removed from the 23A2 cultures at various times after transfection with the H-*ras* oncogene. 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.

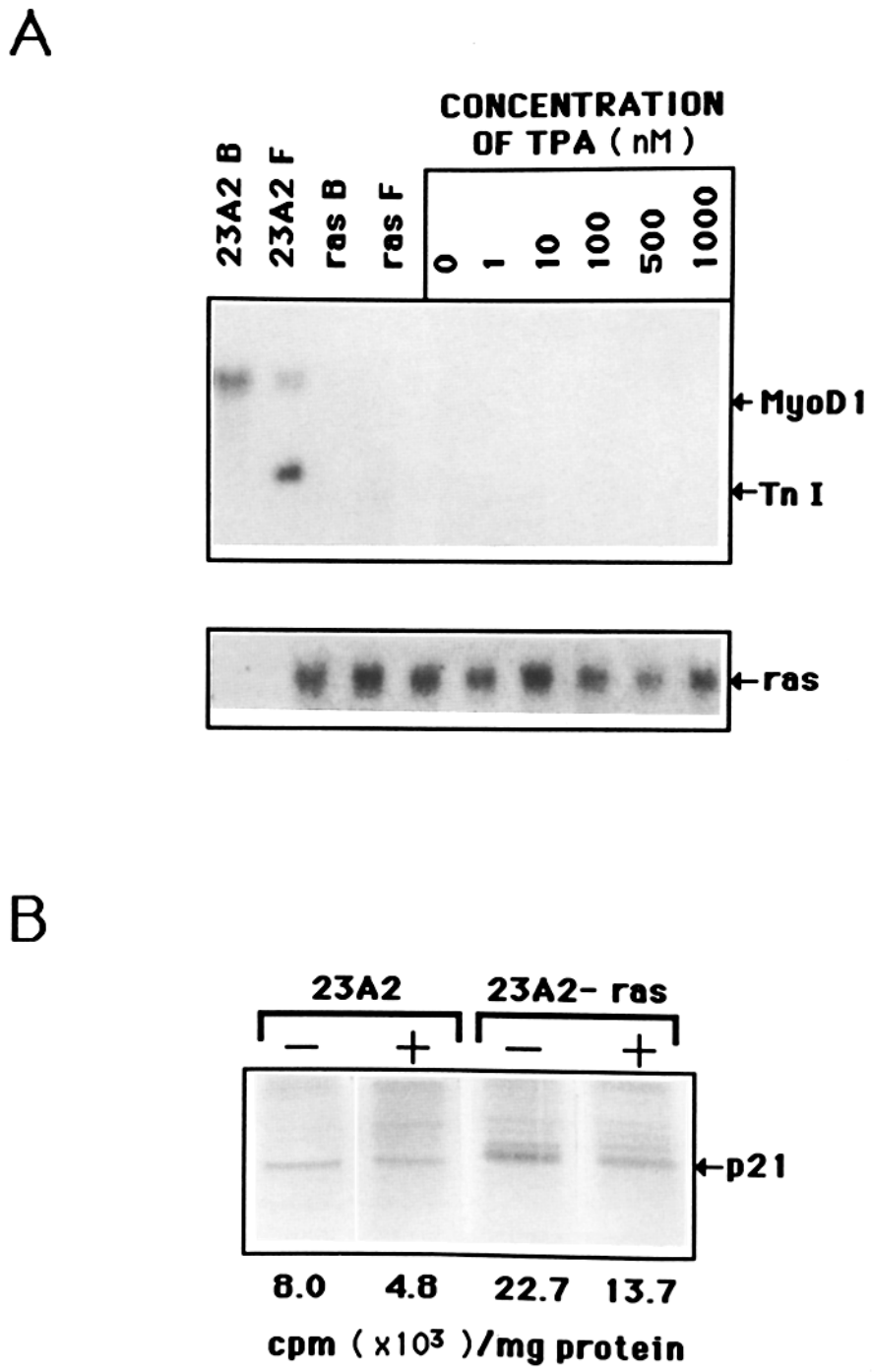
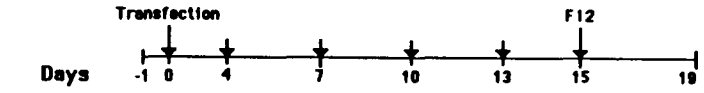


Figure 7. Northern blot analysis of total RNA isolated from TPA-treated 23A2-*ras* cells. Cultures were fed differentiation-inducing medium supplemented with concentrations of TPA from 1 nM to 1 μM. 15 μg of total RNA were electrophoresed through an agarose/formaldehyde gel, transferred to nitrocellulose, and probed for *MyoD1* and TnI mRNAs as described in Materials and Methods. The filter was subsequently stripped of the previous probes by washing in 0.1× SSC containing 0.1% SDS for 30 min at 90–100°C with one change of wash solution and then reprobed for the expression of the transfected H-*ras* oncogene (*ras*). Control lanes contain 15 μg of total RNA from 23A2 myoblasts (*23A2 B*), 23A2 myofibers (*23A2 F*), proliferating 23A2-*ras* cells (*ras B*), and differentiation-induced 23A2-*ras* cells (*ras F*). (*B*) Immunoprecipitation of total *ras* p21 from 23A2 myoblasts (*23A2*) and the differentiation-defective 23A2 pT249a2 cells (*23A2-ras*). Cells were metabolically labeled with [^{35}S]methionine in the presence (+) or absence (−) of 500 nM TPA. *ras* p21 was immunoprecipitated from labeled cell extracts using the Y13-259 antibody (20) and electrophoretically resolved on a denaturing polyacrylamide gel. Quantitation of the *ras* p21 in each sample was achieved by excising the relevant portion of each lane and counting in a scintillation counter. Background counts obtained from parallel lanes precipitated without the Y13-259 antibody (not shown) were subtracted from each value before expression as cpm/mg protein. The values reported for each sample represent the average of three independent determinations.

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



Groups		No. of myogenic colonies / total no. of colonies			
		Expt.1	Expt.2	Expt.3	Average[b]
Control	A	104/138 (.75)[a]	221/238 (.93)	312/349 (.89)	.86
	B TPA	109/171 (.64)	189/252 (.75)	282/333 (.85)	.75
ras	C	19/206 (.09)	23/262 (.09)	51/359 (.14)	.11
	D TPA	37/196 (.19)	57/222 (.26)	76/327 (.23)	.23
	E TPA	64/197 (.33)	103/232 (.44)	103/321 (.32)	.36
	F TPA	95/182 (.52)	133/246 (.54)	111/195 (.57)	.54
	G TPA	54/236 (.23)	96/266 (.36)	114/251 (.45)	.35
	H TPA	19/216 (.09)	42/274 (.15)	59/250 (.24)	.16

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.

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

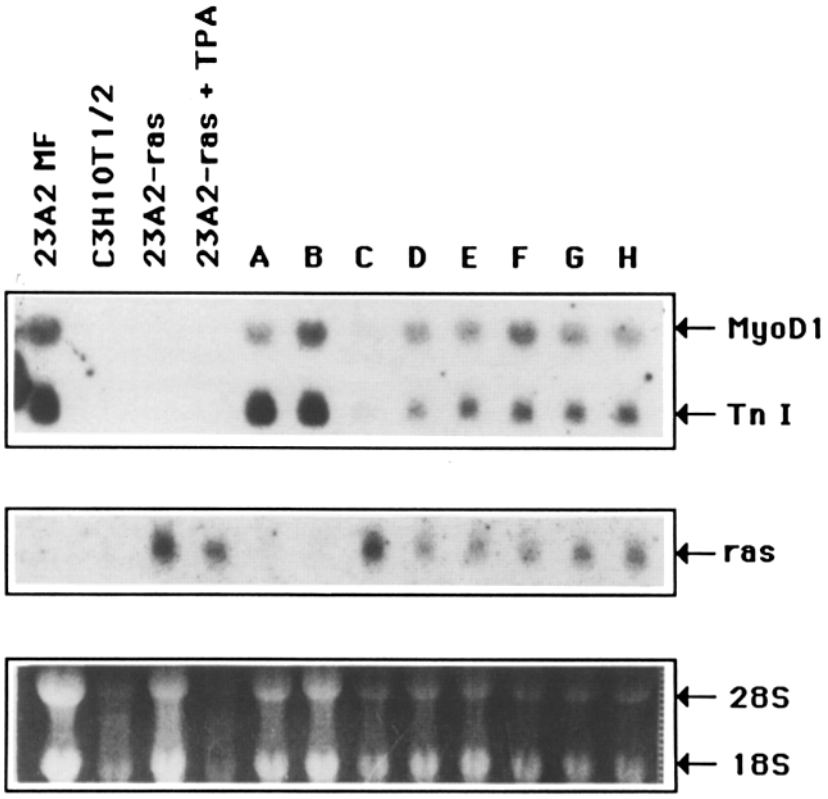


Figure 9. Northern blot analysis of total RNA isolated from Groups A–H of Exp. 3 in Fig. 8. 15 μg of RNA from each experimental group were electrophoresed through an agarose/formaldehyde gel, transferred to nitrocellulose, and probed for *MyoD1* and TnI mRNAs as described in Materials and Methods. The 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 pT249a2 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.

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 cells 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 N-*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. Taparowsky, 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. Taparowsky, 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 downregulation 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.

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