Rapid Activation and Down-Regulation of Protein Kinase C α in 12-*O*-Tetradecanoylphorbol-13-acetate-induced Differentiation of Human Rhabdomyosarcoma Cells[1]

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Abstract

Human rhabdomyosarcoma RD cells express the myogenic regulatory factors MyoD and myogenin but differentiate spontaneously very poorly. Prolonged treatment of RD cells with the protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces growth arrest and myogenic differentiation as shown by the accumulation of α-actin and myosin light and heavy chains, without affecting the expression of MyoD and myogenin. In this study, we show that short-term phorbol ester treatment of the cultures is sufficient to trigger myogenic differentiation but not growth arrest. Furthermore, PKC inhibitors, such as staurosporine or calphostin C, prevent TPA-induced differentiation but not cell growth arrest. These data suggest that the two events are mediated by different pathways; a possible interpretation is that the activation of one or more PKC isoforms mediates the induction of differentiation, whereas the down-regulation of the same or different isoforms mediates the growth arrest. To address the mechanism whereby TPA affects cell growth and differentiation in RD cells, we first analyzed PKC isoenzyme distribution. We found that RD cells express the α, β_1, γ, and ζ PKC isoenzymes. Only the α isoform is exclusively found in the soluble fraction, but it translocates to the membrane fraction within 5 min of TPA treatment and is completely down-regulated after 6 h. The other isoenzymes are found associated to both the soluble and the particulate fractions and are down-regulated after long-term TPA treatment. By immunofluorescence analysis, we show that the PKC α down-regulation is specific for those cells that respond to TPA by activating the muscle phenotype. We propose that TPA-induced differentiation in RD cells is mediated by the transient activation of PKC α, which activates some of the intracellular events that are necessary for MyoD and myogenin transacting activity and for the induction of terminal differentiation of RD cells. By contrast, the constitutively active β_1 and ζ are responsible for the maintenance of cell growth, and their down-regulation is responsible for long-term TPA-induced cell growth arrest.

Introduction

Rhabdomyosarcomas are a class of myoblast-derived soft sarcomas that primarily affect children and young adults (1). They undergo an abortive myogenic differentiation, although they express some of the MRFs[3] (2, 3). These factors belong to the helix-loop-helix family of transcription factors and are characterized by the ability, upon transfection, to convert nonmyogenic cells to the muscle phenotype (4–8). Because of these features, they are considered "master genes," and their expression in cells where no or very little myogenic differentiation occurs, such as rhabdomyosarcomas, appears to be paradoxical. Similar to the *in vivo* counterpart, the human rhabdomyosarcoma cell line RD undergoes a limited and abortive myogenic differentiation, although it expresses the MRFs, MyoD and myogenin (9–12). Treatment of RD cells with phorbol esters, such as TPA, induces growth arrest and myogenic differentiation without modifying the pattern of expression of the regulatory genes (10, 11). The myogenic program is activated in the RD nuclei when RD cells are fused with C3H10T½ murine fibroblasts (12). These data suggest that the suppression of differentiation in these cells is due to mechanisms that interfere with the activity of the MRFs, downstream from their expression. This suppression can be removed by TPA, probably by activation of events that results in the formation of an active transcriptional complex containing MyoD and/or myogenin.

Since TPA is a potent activator of PKC (13), it is conceivable that alterations in the activity of this enzyme can play a major role in controlling the differentiation of RD cells. The PKCs constitute a family of serine/threonine protein kinases that appear to be involved in different cellular processes, such as growth and differentiation (14). To date, 11 different isoforms have been identified and cloned. They can be divided into two major groups: the conventional calcium-, phospholipid-dependent PKCs (conventional PKC α, β_1, β_2, and γ) and the novel calcium-independent PKCs (novel PKC δ, ε, ζ, η, θ, λ, and μ; reviewed in Refs. 14 and 15). Some of the novel PKC isoforms, such as the ζ, λ, and μ isoforms, are TPA independent (14, 16, 17). These different isoenzymes have been shown to exhibit differential tissue distribution and cellular localization; whereas the

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[3] The abbreviations used are: MRFs, myogenic regulatory factors; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate.

α, β, δ, and ζ isoenzymes are widespread, others, such as γ, η, and θ, are restricted to one or few tissues (18), and multiple isoenzymes are often found in a single cell type (18, 19). To date, very little is known about substrate specificity among the different isoforms, but increasing data are being collected about specific biological roles in different systems (15). For example, it has been shown recently that PKC α and δ are involved in the TPA-induced myeloid differentiation, whereas β_2 is involved in the control of proliferation in erythroleukemia cells (20, 21).

To address the mechanism whereby TPA affects differentiation and growth of RD cells, we analyzed the involvement of the individual PKC isoenzymes. We present evidence that the transient activation of PKC α is responsible for the induction of differentiation, probably mediating posttranslational modifications necessary for the induction of the myogenic program. By contrast, the down-regulation of PKC β_1 and ζ may be relevant to the TPA-induced growth arrest.

Results

Phorbol Ester-induced Differentiation Requires a Short-term Treatment and Activation of Protein Kinase C. Myogenic differentiation of RD cells was induced by a short exposure to the phorbol ester PDBu. PDBu has similar biological effects on cultured cells but is more hydrophilic than TPA and, therefore, it is easier to remove from the cells (22). RD cells were treated with 100 nM PDBu for 2 h and then washed and cultured for an additional 6 days without PDBu. The cells were then fixed and analyzed for myosin expression by using the anti-skeletal myosin heavy chain mAb MF20 in an immunoperoxidase assay (Fig. 1). Total number of nuclei and the number of nuclei in myosin-positive cells were evaluated in each condition from different experiments, and the data are summarized in Table 1. The phorbol ester-induced decrease in number of total nuclei is due to inhibition of cell growth since [^{3}H]thymidine incorporation is inhibited and cell death rate is not affected in this condition (10). Moreover, the induction of myogenic differentiation is likely to be at the transcriptional level since, by Northern blot analysis, accumulation of muscle-specific transcripts is observed as early as after 24 h of TPA treatment (11). As shown in Table 1, a short-term (2-h) PDBu treatment is sufficient to significantly increase the number of myosin-positive cells when compared to control cells (Fig. 1, *a* and *b*; Table 1). By contrast, this short-term PDBu treatment does not inhibit cell proliferation, while long-term (6-day) treatment does (Fig. 1, *b* and *c*; Table 1). As a result, both short- and long-term PDBu treatments result in the same number of myosin-positive cells; however, these cells represent a small percentage of the total cell population in the 2-h PDBu experiments and a much larger proportion in long-term PDBu treatment experiments. These data suggest that the early events induced by PDBu are responsible for the expression of the differentiated phenotype, whereas later events, induced by prolonged PDBu treatment, are responsible for the inhibition of cell proliferation.

An important question is whether growth arrest alone will result in an increased number of myosin-expressing cells. To address this question, RD cells were treated for 2 h with mitomycin C, a known inhibitor of cell proliferation, and then the cells were analyzed for myosin expression after an additional 6 days in culture, in the presence or absence of TPA. As shown in Table 1, no increase of myosin-positive cells in mitomycin-arrested cells or enhancement of the TPA-induced differentiation was observed. The proportion of myosin-positive cells, in the absence of TPA, appears slightly higher than in control cells, since the entire cell population is irreversibly growth arrested.

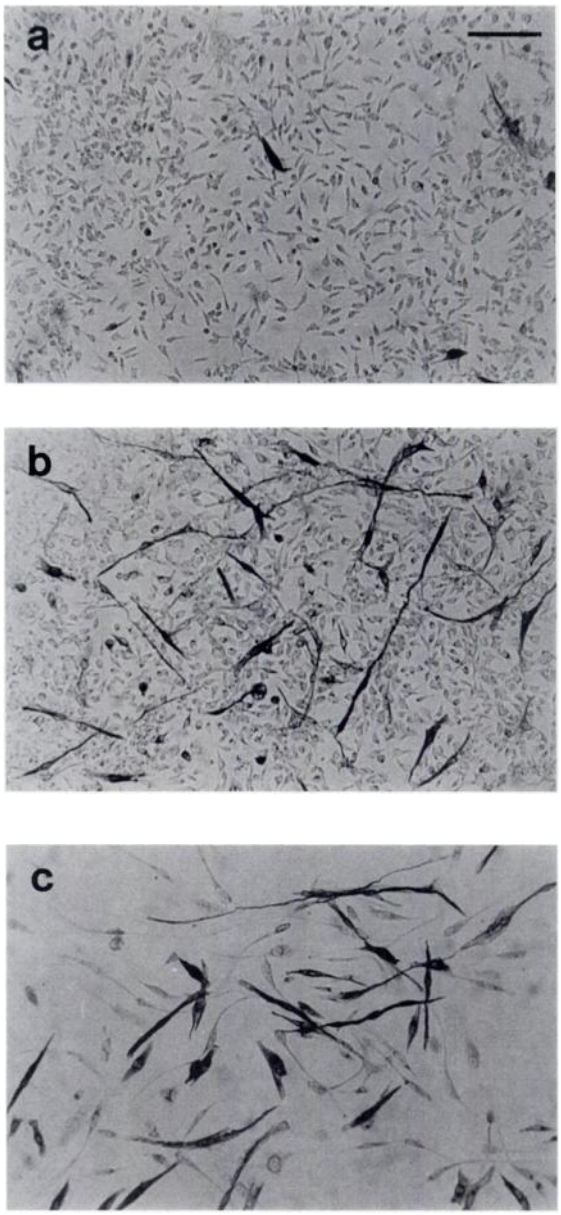

Fig. 1. Immunostaining of myosin heavy chain with the MF20 mAb in RD cells cultured for 6 days. *a*, control cells; *b*, cells treated for 2 h with 100 nM PDBu and then cultured for 6 days in the absence of PDBu; *c*, cells treated for 6 days with 100 nM PDBu. *Bar*, 50 μm.

PDBu, as well as TPA, is known to act through PKC. Phorbol esters initially stimulate PKC, whereas they down-regulate the enzyme after prolonged exposure (23, 24). To investigate whether the TPA-induced differentiation is dependent upon PKC activation or down-regulation, we treated RD cells with known PKC inhibitors. We used two different PKC inhibitors (25). Staurosporine was used at a

Table 1 Effect of PDBu and Mitomycin C treatment on proliferation and differentiation of RD cells

RD cells cultured for 6 days at the indicated conditions were evaluated for total number of nuclei and the number of nuclei in myosin-positive cells. The values represent the average of at least three different experiments (± SD).

Treatment	Total nuclei	Nuclei in myosin-positive cells	
	Nuclei x 10^{-3}/cm^2	Nuclei x 10^{-3}/cm^2	%
Control	136.0 ± 25.0	1.2 ± 0.60	0.8
PDBu, 2h	138.0 ± 24.0	4.3 ± 0.70	3.1
PDBu, 6 days	13.0 ± 3.0	4.2 ± 0.80	33.0
Mitomycin C, 2 h	3.5 ± 0.3	0.1 ± 0.01	2.5
Mitomycin C, 2h, + TPA	3.7 ± 0.5	0.8 ± 0.01	22.0

concentration where it preferentially inhibits PKC (1 nM). Calphostin C (100 nM), a more specific PKC inhibitor, was also used. The cells were treated with these inhibitors in the presence or absence of TPA, and after 6 days in culture, were analyzed for the expression of skeletal myosin by immunocytochemistry. Total number of nuclei and the proportion of nuclei in myosin-positive cells are summarized in Fig. 2. The results demonstrate that both PKC inhibitors, by themselves, partially induce growth arrest but prevent the TPA-induced myogenic differentiation. Taken together (Figs. 1 and 2), these data demonstrate that the induction of differentiation and the growth arrest effects are independent events, regulated by different pathways. TPA-induced differentiation may require activation of PKC, whereas the inhibition of cell proliferation may be mediated by its down-regulation.

TPA Induces a Rapid Translocation of PKC α to the Particulate Fraction. To determine which PKC isoform(s) may mediate the differentiation response in RD cells, we first determined which isoenzymes are expressed in these cells. Western blot analysis revealed that the RD cells contained PKC α, β_1, γ, and ζ (Fig. 3, *Lanes 1*). The apparent molecular weights of these immunoreactive bands were similar to those of lysates from mouse brain (Fig. 3, *Lanes 2*). The specificity of the bands was ensured by preincubating the antisera with an excess of the specific antigenic peptides (data not shown). These findings provide evidence that PKC α, β_1, γ, and ζ are expressed in the RD cells.

It is known that PKCs in their inactive form are found in the soluble fraction, whereas the active forms are associated with the particulate fraction (26, 27). We, therefore, treated the cells with TPA for different periods of time and analyzed the distribution of each isoform in the soluble and particulate fractions. Fig. 4 shows that the α isoform is accumulated in the soluble fraction in untreated cells but translocates to the particulate fraction within 5 min of TPA treatment. In this particulate fraction, a lower molecular weight band (M_r 72,000) is also evident in the 1-h treatment sample. The appearance of this M_r 72,000 form is blocked by preincubation of the antisera with the antigenic peptide (data not shown), thus suggesting that this band represents a proteolytic fragment. The presence of the α isoform in the particulate fraction is maintained for up to 6 h, after which it disappears from this compartment (Fig. 5). These results were obtained by using two different anti-PKC α-specific antisera (see "Materials and Methods").

Compared to PKC α, PKC β_1 is located in both the soluble and the particulate fraction of RD cells (Fig. 5). Within 24 h

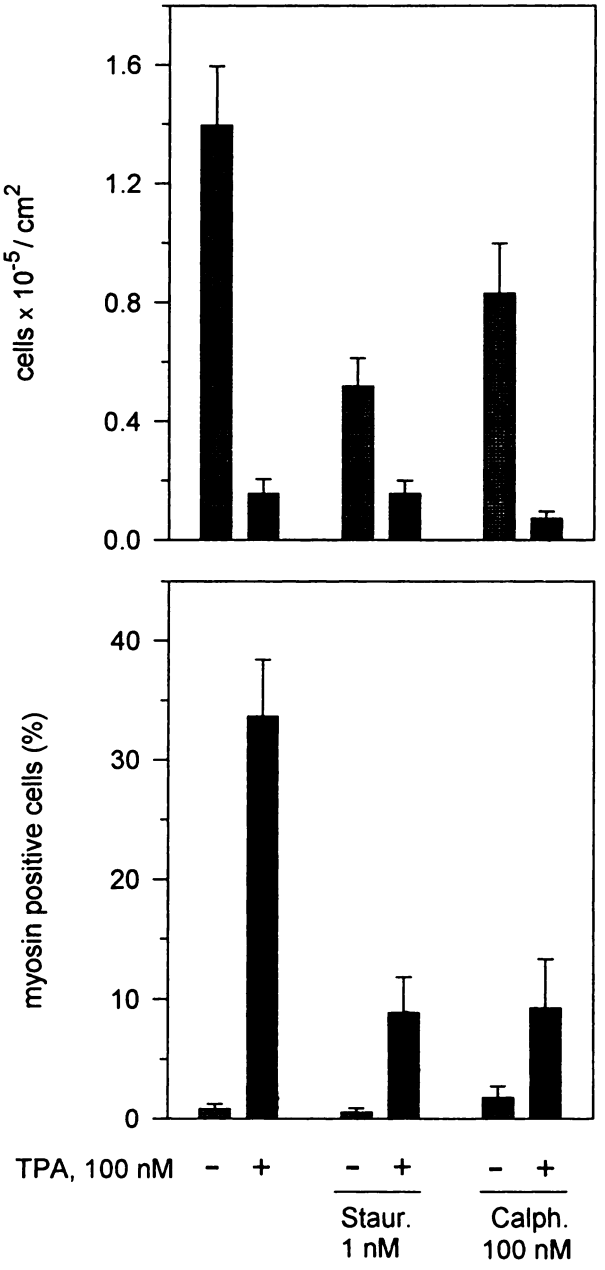

Fig. 2. Effect of PKC inhibitors on proliferation and differentiation of RD cells. RD cells were cultured for 6 days in the absence (*hatched bars*) or presence (■) of 100 nM TPA, with 1 nM staurosporine or 100 nM calphostin C as indicated. Total number of nuclei at the end of incubation was evaluated for each condition (*top panel*). The percentage of nuclei in myosin-positive cells was established by MF20 immunoperoxidase assay. Data represent the average of at least three different experiments; *bars*, SD.

of TPA treatment, the accumulation of the β_1 isoform in the particulate fraction is almost unaffected, while it slowly decreases in the soluble fraction. By contrast, prolonged treatment with TPA (72 h) induces a significant loss of PKC β_1 in the particulate fraction, but, at this time of treatment, it is still detectable in the soluble fraction (Fig. 5). In this fraction, a lower molecular weight band is also detectable, whose nature is still not understood. The γ isoform is

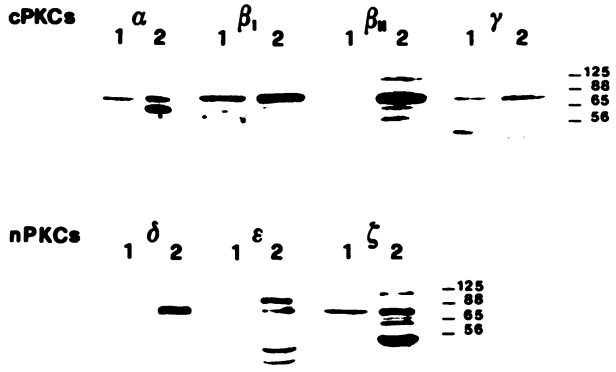

Fig. 3. Western blot analysis of PKC isoforms in total RD cell lysate. Equal volumes of PKC-enriched DEAE-eluted sample (prepared as described in "Materials and Methods") were loaded on each lane. *Lane 1,* RD cells; *Lane 2,* total brain lysate (used as control). The specificity of the bands was determined by electrophoretic mobility and competition using an excess of the specific immunogen peptide (data not shown).

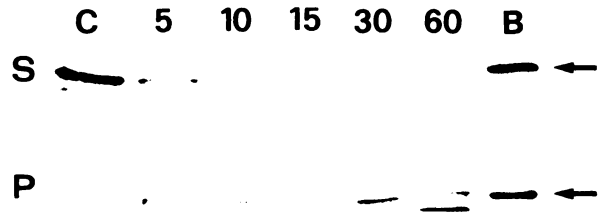

Fig. 4. Western blot analysis of fractionated lysates from RD cells cultured for 5 min to 1 h in the presence of TPA. The blots were incubated with the anti-PKC α antiserum. *Arrow,* the M_r 80,000 PKC α immunoreactive band. *S,* soluble fraction; *P,* particulate fraction.

expressed at a very low level and is not affected by TPA treatment (not shown). The ζ isoform, which is known to be TPA insensitive, is also found to be present in both the particulate and soluble fraction of control cells. TPA treatment induces a loss of PKC ζ in the particulate and in the soluble fraction by 24 and 72 h, respectively (Fig. 5). Densitometric analysis of these results is shown in Fig. 6. Only the specific bands indicated by the *arrows* in Figs. 4 and 5 were included in this analysis. For each PKC isoform, the densitometric data of each time course (soluble or particulate) were normalized by assigning a value of 100% to the densest band. The observed association of PKC β_1 and ζ to the particulate fraction in untreated cells suggests that these isoforms may be constitutively active and may, therefore, account for the high proliferation rate of these cells. The slow and progressive loss of these isoenzymes in TPA-treated cells (relative to PKC α) may represent the late event responsible for the TPA-induced growth arrest.

Down-Regulation of PKC α Is Specific for the Differentiated Rhabdomyoblasts. Since only a part of the RD cell population differentiates after TPA treatment (10, 11), we performed double immunofluorescence analysis to establish a possible correlation between the TPA-induced differentiation and the effect on PKC α expression. RD cells were cultured for 6 days in the presence or absence of TPA and analyzed for the expression of PKC α and skeletal myosin by double immunofluorescence. Fig. 7 shows that PKC α is localized in the nuclei of control RD cells (Fig. 7*a*). However, following TPA treatment, the PKC α immunostaining is completely abolished in the myosin-positive cells (Fig. 7, *c* and *d*). In particular, the inhibition of PKC α immunostaining is specific for those cells that differentiate after TPA treatment since all, and only, the myosin-positive cells are PKC α negative. In RD cells, PKC α may be associated with the nucleoplasm since we find its accumulation in soluble fraction by Western blot, as it has already been described in other cell systems (28).

Discussion

PKC is known to be involved in a variety of pleiotropic processes, such as cell proliferation and differentiation (14). TPA represents one of the major activators of this family of kinases, but long-term TPA treatment induces desensitization and down-regulation of PKC (23). In many cell types, including fibroblasts (23, 29), T lymphocytes (24) and myoblasts (30, 31), TPA induces cell proliferation. By contrast, TPA has been shown to induce differentiation in leukaemia cell lines, such as HL60 (32) or K562 (33), and in the human rhabdomyosarcoma cell line RD (10). These opposite proliferative and differentiative effects induced by TPA may depend on the different cellular environment. Since TPA acts via PKC and up to eleven different isoenzymes have been described to date (14, 15, 18), it is conceivable that the different effects induced by TPA in different cell types may depend on the PKC isoenzymes expressed. The activation or down-regulation of specific isoforms may selectively mediate these proliferative or differentiative effects.

The human rhabdomyosarcoma RD cells undergo limited and abortive myogenic differentiation, although they express correctly the MRFs MyoD and myogenin (9, 11, 12). Furthermore, both MyoD (12) and myogenin[4] are able to bind their specific DNA consensus sequences, but their transacting activity is abolished. TPA treatment induces growth arrest and myogenic differentiation without modifying the expression (11) and the DNA-binding activity[4] of these factors. It is known that the transacting activity of these factors depends on their interaction with other DNA-binding factors, some of which have been identified and others are still unknown, that enhances their transcriptional activity (34–36). It can be postulated that posttranslation modifications, induced by TPA via specific PKC isoenzymes, may trigger the formation of an active multicomponent transcriptional complex.

We show in this study that short-term treatment with phorbol esters is sufficient to activate myogenic differentiation, but not growth arrest, in the RD cell population. We do not know whether the entire cell population or only those cells that are not committed to differentiate continue to proliferate after removal of the phorbol esters. Although the percentage of differentiated cells (relative to the total cell population) is different as the result of short- *versus* long-term phorbol ester treatment, their absolute number is equivalent. It is possible that only those cells that are in a particular status (*i.e.,* cell cycle stage) respond to the phorbol esters activating the differentiation program. However, we can exclude that it may be a consequence of previous exit from the cell cycle since mitomycin-arrested RD cells do not spontaneously differentiate or respond more effi-

[4] M. Bouché, unpublished observations.

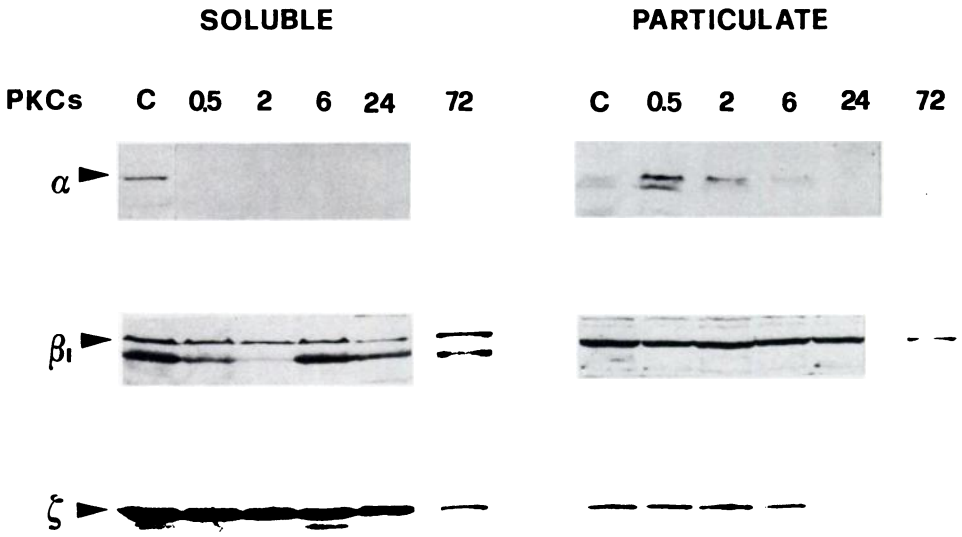

Fig. 5. Western blot analysis of fractionated lysates from RD cells treated for different periods of time with TPA and incubated with the specific anti-PKC antisera as indicated. *Arrows,* the specific PKC band for each blot.

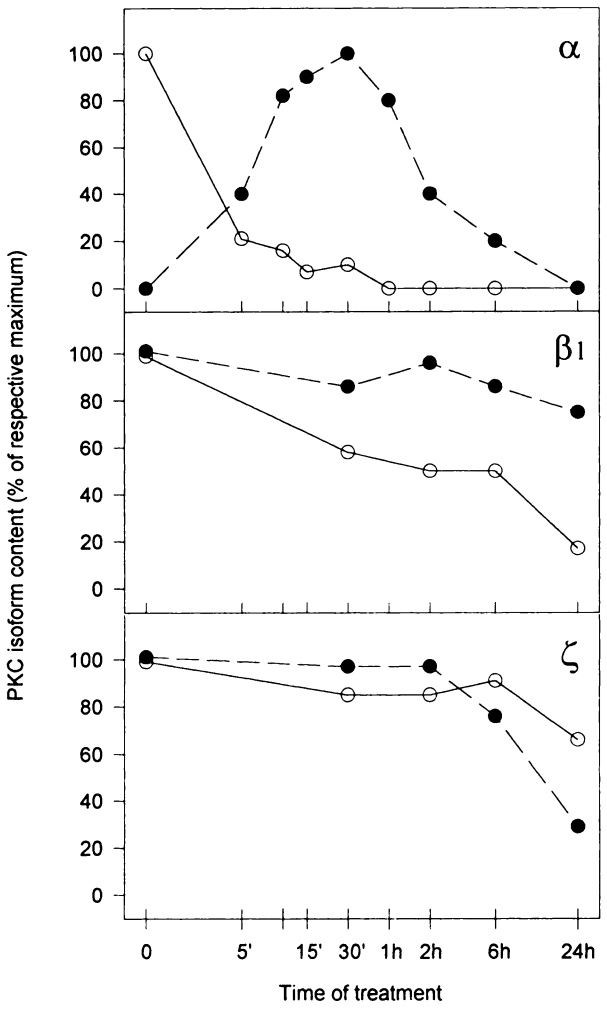

Fig. 6. Densitometric analysis of the Western blots shown in Figs. 4 and 5. Only the specific bands (indicated by the *arrows* in Figs. 4 and 5) were included in this analysis. The densest band for each time course (soluble or particulate) was taken as 100%. ○, soluble fraction; ●, particulate fraction.

ciently to TPA treatment. The possibility of heterogeneity of the cell population seems to be ruled out by the observation that repeated subcloning of RD cells yields constant numbers of cells differentiating in response to TPA (10). Furthermore, the PKC inhibitors staurosporine or calphostin C prevent TPA-induced differentiation but not the inhibition of cell proliferation. These data suggest that activation of PKCs (an early TPA-induced event) is involved in the TPA-induced differentiation of RD cells, whereas their down-regulation (a late TPA-induced event) may be responsible for the growth arrest. Uncoupling of growth arrest and differentiation in dexamethasone-treated RD cells has also been reported (37). Two possibilities can be hypothesized: (*a*) specific PKC isoforms are responsible for the two types of response; and (*b*) the activation of the same PKC isoform(s) is involved in the regulation of the differentiation pathway, whereas its down-regulation is responsible for the inhibition of cell proliferation observed as a result of long-term phorbol ester treatment. We show that RD cells express four (α, β_1, γ, and ζ) of the eight PKC isoforms that we have screened. Among these, the α isoenzyme is localized in the soluble fraction of the nuclei of control RD cells, indicating that it is probably in its inactive form (26, 27). By comparison, we find that the β_1 and ζ isoenzymes are present in both soluble and particulate fraction. In particular, these two isoforms are prominent in the particulate fraction, indicating that they are probably constitutively active in nonstimulated cells in our culture conditions. However, both these isoenzymes are down regulated after long-term TPA treatment, although with different kinetics, with no evidence of any TPA-induced membrane translocation. In contrast to PKC β_1 and ζ, translocation of PKC α is very rapid, and it occurs within 5 min of TPA exposure. The PKCα translocation is maintained for up to 6 h; then the isoenzyme is no longer detectable in either the soluble or the particulate fraction. These data suggest that TPA treatment induces transient activation of this isoform, which is controlled at the protein level since the PKC α mRNA accumulation is not affected by prolonged TPA treatment (10). Since myogenic differentiation of RD cells is activated by early events induced by TPA, we propose that the transient activation of PKC α mediates the differentiative

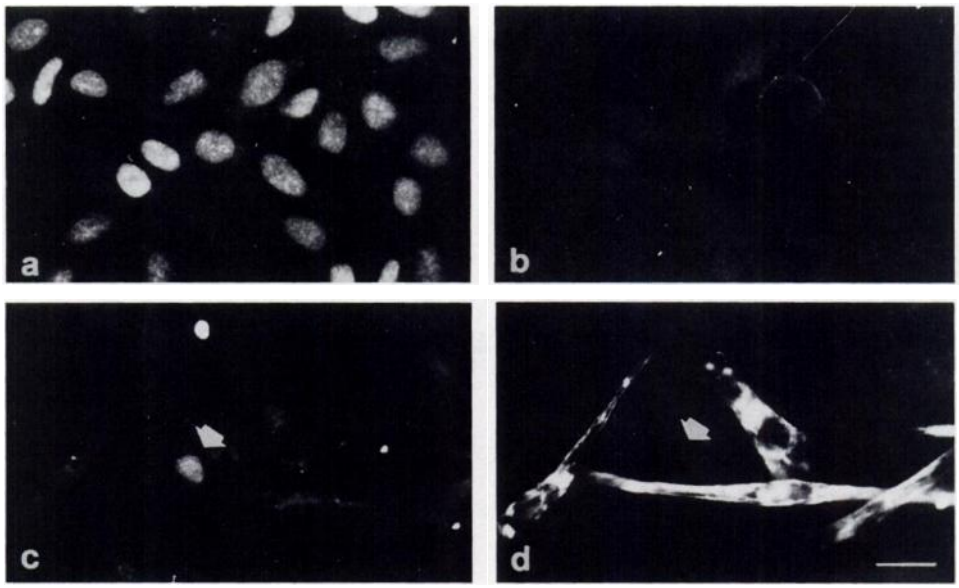

Fig. 7. Double immunofluorescence analysis of RD cells cultured for 6 days in the absence (*a* and *b*) or presence (*c* and *d*) of TPA and stained with the anti-PKC α-specific antiserum (*a* and *c*) and the myosin heavy chain MF20 mAb (*b* and *d*). *Arrow* (*c* and *d*), one cell that is PKC α positive and myosin negative in the TPA-treated cell population. *Bar*, 10 μm.

effect. Its long-term down-regulation should be a consequence of the induction of the differentiation pathway, since loss of PKC α immunoreactivity is specific for those cells that differentiate upon TPA treatment. The β_1 isoform is normally activated in RD cells, and long-term TPA treatment induces its down-regulation. This event may be responsible for the TPA inhibition of proliferation, and its constitutive activation in control cells may be necessary for continuous growth. It is also conceivable that the long-term down-regulation of the ζ isoform may be the result of pleiotropic effects induced by TPA, probably related to the suppression of cell growth, since this isoenzyme is known to be TPA insensitive (16). Similar differential involvement of PKC α and β isoforms has been described in leukemic cells (20, 21).

Expression of activated Ras in RD cells has been reported (38). This oncogene is known to negatively affect myogenic differentiation, probably inhibiting the expression of the MRFs, through a PKC-mediated mechanism (39–41). Since RD cells express MyoD and myogenin, it is unlikely that the expression of activated Ras is responsible for the suppression of differentiation. However, it has been recently shown that the modulation of gene expression by TPA is mediated by specific PKC isoenzyme(s) in a Ras-dependent manner (42). Surprisingly, overexpression of PKC α suppresses this pathway (42). The TPA-induced PKC α transient activation may play an equivalent role in RD cells by suppressing Ras activity and, thereby, permitting differentiation to occur.

Our results indicate that the transient activation of PKC α may represent a possible molecular mechanism by which TPA induces myogenic differentiation. It has been shown that PKC-mediated phosphorylation of MyoD and myogenin abolishes their binding activity (43). Since we found no evidence that MyoD or myogenin binding activity was affected by phorbol ester treatment,[4] it is unlikely that PKC phosphorylation of the MRFs is the regulatory event in this process. It has also been shown that the active hypophosphorylated Rb protein directly interacts with the MRFs and mediates muscle cell cycle withdrawal and activation of myogenic differentiation; furthermore, Rb is expressed in RD cells and interacts with the MRFs (44). Our preliminary results indicate that TPA does not modify the expression of Rb.[4] However, overexpression of p107, a similar protein of the pocket family, induces cell cycle withdrawal and myogenic differentiation in RD cells (45). We suggest that the activity of these or other factors may be controlled, directly or indirectly, by PKC α-mediated phosphorylation events, thus inducing the transcriptional activity of the MRFs.

Materials and Methods

Cell Culture. RD human embryonal rhabdomyosarcoma cells, obtained from the American Type Culture Collection (Rockville, MD), were cultured as described (10, 11). The cells were periodically cloned and used between the 4th and the 18th passage. Cells were grown (5×10^3 cells/cm^2) in DMEM (Hyclone) supplemented with 4 mM glutamine, 40 μg/ml gentamicin, and 10% FCS in a humidified atmosphere of 5% CO_2 at 37°C. TPA or PDBu, dissolved in ethanol at 0.1 mM, was added to the cells 24 h after plating (final concentration, 100 nM); ethanol alone was added to companion cells used as control.

Antisera and Antibodies. Several different primary antisera were used in this study. The PKC α antiserum (Boehringer-Mannheim) was raised against amino acids 313–326 of the human isoenzyme. A different PKC α and γ antisera were raised against synthetic peptides.[5] The PKC α peptide included amino acids 305–326 (FEKAKLGPAGNKVISEDRRQ) from bovine sequence, whereas the PKC γ peptide included amino acids 302–322 (FEACNYPLELYEVSRTGPSSS) from the human sequence (46). The peptides were coupled to bovine thyroglobulin by carbidiimide, and antisera to these two isoforms were raised in rabbits as described (18). Separation of proteins from platelet lysates by a series of chromatographic steps ending with hydroxylapatite chromatography revealed that the PKC α bound

[5] W. C. Wetsel, unpublished observations.

only material that eluted in the position of human PKC α, and the PKC γ antiserum recognized only material that eluted in the position of human PKC γ. These respective antisera did not appear to bind any other isoforms. On a Western blot, these immunoreactive species were approximately M_r 80,000 in size, and the immunostaining could be blocked with the appropriate antigenic peptide. Antisera to β_1, β_2, δ, ε, and ζ were raised in rabbits against unique amino acid sequences located in the extreme carboxy terminals of the different enzymes (18). A monoclonal antibody to the myosin heavy chain (MF20 antibody), which recognizes all sarcomeric myosins, was used to determine the differentiation state of the human RD. This antibody was kindly provided by Dr. D. A. Fischman (Cornell University Medical College, New York, NY, Ref. 47).

Immunocytochemistry. The RD cells were immunostained with the PKC α antisera and the myosin heavy chain antibody by a double immunofluorescence procedure as described (11). After 6 days in culture, the RD cells were fixed and permeabilized in ethanol:acetone (1:1) at −20°C for 20 min. The cells were incubated for 30 min in Dulbecco's PBS containing 1% BSA. Afterwards, the cells were coincubated for 1 h with one of the different PKC α antisera and the MF20 antibody against the skeletal myosin heavy chain. Finally, the cells were incubated with fluorescein-conjugated goat anti-rabbit IgG antisera and a rhodamine-conjugated goat anti-mouse IgG (both from Cappel). Cells were mounted in 75% glycerol in Tris (pH 9.0) and evaluated under an epifluorescence Zeiss microscope.

The differentiation status of the RD cells was determined by immunostaining with the MF20 antibody. In this case, a biotin-conjugated goat anti-mouse IgG antiserum (Cappel) was used as the secondary antiserum. After incubation with this antiserum and washing, the cells were incubated with horseradish peroxidase-conjugated streptavidin (Zymed). Diaminobenzidine (0.4 mg/ml; Sigma Chemical Co.) was used as the substrate for peroxidase to visualize the immunostained cells.

Preparation of Cellular Extracts. The cell pellet were resuspended in ice-cold homogenization buffer (H-buffer) containing 20 mM Tris (pH 7.5), 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 5 mM DTT, 200 μg/ml leupeptin, 10 mg/ml trasylol, and 3.5 mg/ml phenylmethylsulfonyl fluoride and sonicated. By this procedure, approximately 97% of the nuclei were disrupted, as shown by microscopic analysis.

When total cell extracts were required, cells were homogenized in the H-buffer containing 0.1% Triton X-100; then the homogenate was incubated for 30 min on ice with repeated vortexing and centrifuged at 15,000 × *g* for 15 min. An aliquot of the supernatant was used for protein determination by the Comassie plus protein assay reagent (Pierce) according to the manufacturer's instructions, while the remainder was subjected to DEAE Sepharose Fast-Flow (Pharmacia) chromatography as described (48). The eluted material is referred to as total lysate. For subcellular fractionation, cell homogenates (without Triton X-100) were centrifuged at 100,000 × *g* for 30 min at 4°C in a Beckman TL-100 centrifuge. The supernatant constitutes the soluble fraction. The pellet was resuspended in H-buffer containing 0.1% Triton X-100, extracted as described above, and centrifuged at 15,000 × *g* for 15 min. This supernatant is referred to as particulate fraction.

Western Blot Analysis. Total lysates or soluble and particulate fractions were mixed with Laemmli sample buffer, boiled for 5 min, and loaded onto 10% SDS-polyacrylamide gels (49). The gels were blotted onto nylon filter (Hybond C; Amersham) and probed with the appropriate specific antisera (18). Peroxidase-conjugated goat anti-rabbit IgG (Cappel) was used as secondary antibody, and detection was performed by the ECL method (Amersham) according to the manufacturer's instructions. Scanning densitometry was performed on fluorograms of the Western blot with a laser densitometer (Ultroscan XL; Pharmacia LKB). Data were analyzed using the GSXL program (Pharmacia LKB).

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References

1. Malone, M. Soft tissue tumours in childhood. Histopathology, *23:* 203–216, 1993.

2. Dias, P., Parham, D. M., Shapiro, D. M., Webber, B. L., and Houghton, P. J. Myogenic regulatory protein (MyoD1) expression in childhood solid tumors: diagnostic utility in rhabdomyosarcoma. Am. J. Pathol., *137:* 1283–1291, 1990.

3. Tonin, P. N., Scrable, H., Shimada, H., and Cavenee, W. K. Muscle-specific gene expression in rhabdomyosarcomas and stages of human fetal skeletal muscle development. Cancer Res., *51:* 5100–5106, 1991.

4. Davis, R. L., Weintraub, H., and Lassar, A. B. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell, *51:* 987–1000, 1987.

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

6. Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., and Arnold, H. H. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T½ fibroblasts. EMBO J., *8:* 701–709, 1989.

7. Rhodes, S. J., and Konieczny, S. F. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev., *3:* 2050–2061, 1989.

8. Miner, J. H., and Wold, B. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proc. Natl. Acad. Sci. USA, *87:* 1089–1093, 1990.

9. Hiti, A., Bogenmann, E., Gonzales, F., and Jones, P. A. Expression of the MyoD1 muscle determination gene defines differentiation capability but not tumorigenicity of human rhabdomyosarcomas. Mol. Cell. Biol., *9:* 4722–4730, 1989.

10. Aguanno, S., Bouché, M., Adamo, S., and Molinaro, M. TPA induced differentiation of a human rhabdomyosarcoma cell line. Cancer Res., *50:* 3377–3382, 1990.

11. Bouché, M., Senni, M. I., Grossi, A. M., Zappelli, F., Polimeni, M., Arnold, H. H., Cossu, G., and Molinaro, M. TPA-induced differentiation of human rhabdomyosarcoma cells: expression of the myogenic regulatory factors. Exp. Cell Res., *208:* 209–217, 1993.

12. Tapscott, S. J., Thayer, M. J., and Weintraub, H. Deficiency in rhabdomyosarcomas of a factor required for MyoD activity and myogenesis. Science (Washington DC), *259:* 1450–1453, 1993.

13. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. Direct activation of calcium-activated phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem., *257:* 7847–7851, 1982.

14. Nishizuka, Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science (Washington DC), *258:* 607–614, 1992.

15. Dekker, L. V., and Parker, P. J. Protein kinase C: a question of specificity. Trends Biochem. Sci., *19:* 73–77, 1994.

16. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. Protein kinase C ζ subspecies from rat brain: its structure, expression, and properties. Proc. Natl. Acad. Sci. USA, *86:* 3099–3103, 1989.

17. Johannes, F. J., Prestle, J., Eis, S., Oberhagemann, P., and Pfizenmaier, K. PKCμ is a novel, atypical member of the protein kinase C family. J. Biol. Chem., *269:* 6140–6148, 1994.

18. Wetsel, W. C., Khan, W. A., Merchenthaler, I., Rivera, H., Halpern, A. E., Phung, H. M., Negro-Vilar, A., and Hannun, Y. A. Tissue and cellular distribution of the extended family of protein kinase C isoenzymes. J. Cell Biol., *117:* 121–133, 1992.

19. Hug, H., and Sarre, T. F. Protein kinase C isoenzymes: divergence in signal transduction? Biochem. J., *291:* 329–343, 1993.

20. Mischak, H., Pierce, J. H., Goodnight, J. A., Kazanietz, M. G., Blumberg, P. M., and Mushinski, J. F. Phorbol ester-induced myeloid differentiation is mediated by protein kinase C-α and -δ and not by protein kinase C-βII, -ϵ, -ζ, -η. J. Biol. Chem., *268:* 20110–20115, 1993.

21. Murray, N. R., Baumgardner, G. P., Burns, D. J., and Fields, A. P. Protein Kinase C isotypes in human erythroleukemia (K562) cell proliferation and differentiation. J. Biol. Chem., *268:* 15847–15853, 1993.

22. Mond, J. J., Feuerstein, N., June, C. H., Balapure, A. K., Glazer, R. I., Witherspoon, K., and Brunswick, M. Bimodal effect of phorbol ester on B cell activation. J. Biol. Chem., *266:* 4458–4463, 1991.

23. Rozengurt, E. Early signals in the mitogenic response. Science (Washington DC), *234:* 161–166, 1986.

24. Berry, N., and Nishizuka, Y. Protein kinase C and T cell activation. Eur. J. Biochem., *189:* 205–214, 1990.

25. Tamaoki, T. Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors. Methods Enzymol., *201:* 340–347, 1991.

26. Kraft, A., and Anderson, W. B. Phorbol esters increase the amount of calcium, phospholipid-dependent protein kinase associated with plasma membrane. Nature (Lond.), *301:* 621–623, 1983.

27. Ito, T., Tanaka, T., Yoshida, T., Onoda, K., Ohta, H., Hagiwara, M., Itoh, Y., Ogura, M., Saito, H., and Hidaka, H. Immunocytochemical evidence for translocation of protein kinase C in human megakaryoblastic leukemic cells: synergistic effects of Ca^{2+} and activators of protein kinase C on the plasma membrane association. J. Cell Biol., *107:* 929–937, 1988.

28. Alessenko, A., Khan, W. A., Wetsel, W. C., and Hannun, Y. A. Selective changes in protein kinase C isoenzymes in rat liver nuclei during liver regeneration. Biochem. Biophys. Res. Commun., *182:* 1333–1339, 1992.

29. Blackshear, P. J., Witters, L. A., Girard, P. R., Kuo, J. F., and Quamo, S. N. Growth factor-stimulated protein phosphorylation in 3T3-L1 cells. J. Biol. Chem., *260:* 13304–13315, 1985.

30. Cohen, R., Pacifici, M., Rubinstein, N., Biehl, J., and Holtzer, H. Effect of a tumor promoter on myogenesis. Nature (Lond.), *266:* 538–540, 1977.

31. Cossu, G., Pacifici, M., Adamo, S., Bouché, M., and Molinaro, M. TPA-induced inhibition of the expression of differentiative traits in cultured myotubes: dependence on protein synthesis. Differentiation, *21:* 62–65, 1982.

32. Fields, A. P., Pettit, G. R., and May, W. S. Phosphorylation of lamin B at the nuclear membrane by activated protein kinase C. J. Biol. Chem., *263:* 8253–8260, 1988.

33. Hocevar, B. A., Morrow, D. M., Tykocinski, M. L., and Fields, A. P. Protein kinase C isotypes in human erythroleukemia cell proliferation and differentiation. J. Cell Sci., *101:* 671–679, 1992.

34. Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N. A new myocyte-specific enhancer-binding factor that recognizes conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol., *9:* 5022–5033, 1989.

35. Cserjesi, P., Lilly, B., Bryson, L., Wang, Y., Sassoon, D. A., and Olson, E. N. MHox: a mesodermally restricted homeodomain protein that binds an essential site in the muscle creatine kinase enhancer. Development (Camb.), *115:* 1087–1101, 1992.

36. Funk, W. D., and Wright, W. E. Cyclic amplification and selection of targets for multicomponent complexes: myogenin interacts with factors recognizing binding sites for basic helix-loop-helix, nuclear factor 1, myocyte-specific enhancer-binding factor 2, and COMP1 factor. Proc. Natl. Acad. Sci. USA, *89:* 9484–9488, 1992.

37. De Giovanni, C., Lollini, P. L., Dolcetti, R., Landuzzi, L., Nicoletti, G., D'Andrea, E., Scotland, K., and Nanni, P. Uncoupling of growth inhibition and differentiation in dexamethasone-treated human rhabdomyosarcoma cells. Br. J. Cancer, *67:* 674–679, 1993.

38. Chardin, P., Yeramian, P., Madaule, P., and Tavitian, A. N-*ras* gene activation in the RD human rhabdomyosarcoma cell line. Int. J. Cancer, *35:* 647–652, 1985.

39. Gossett, L. A., Zhang, W., and Olson, E. N. Dexamethasone-dependent inhibition of differentiation of C2 myoblasts bearing steroid-inducible N-*ras* oncogenes. J. Cell Biol., *106:* 2127–2137, 1988.

40. Sternberg, E. A., Spizz, G., Perry, M. L., and Olson, E. N. A *ras*-dependent pathway abolishes activity of a muscle-specific enhancer upstream from the muscle creatine kinase gene. Mol. Cell. Biol., *9:* 594–601, 1989.

41. Vaidya, T. B., Weyman, C. M., Teegarden, D., Ashendel, C. L., and Taparowsky, E. J. Inhibition of myogenesis by the H-*ras* oncogene: implication of a role for protein kinase C. J. Cell Biol., *114:* 809–820, 1991.

42. Hirai, S., Izumi, Y., Higa, K., Kaibuchi, K., Osada, S., Suzuki, K., and Ohno, S. Ras-dependent signal transduction is indispensable but not sufficient for the activation of AP1/Jun by PKCd. EMBO J., *13:* 2331–2340, 1994.

43. Li, L., Zhou, J., James, G., Heller-Harrison, R., Czech, M. P., and Olson, E. N. FGF inactivates myogenic helix-loop-helix proteins through phosphorylation of a conserved protein kinase C site in their DNA-binding domains. Cell, *71:* 1181–1194, 1992.

44. Gu, W., Schneider, J. W., Condorelli, J., Kaushal, S., Mahdavi, V., and Nadal Ginard, B. Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. Cell, *72:* 309–324, 1993.

45. Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal Ginard, B. Reversal of terminal differentiation mediated by p107 in $Rb^{-/-}$ muscle cells. Science (Washington DC), *264:* 1467–1471, 1994.

46. Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U., and Ullrich, A. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science (Washington DC), *266:* 859–866, 1986.

47. Bader, D., Masaki, T., and Fischman, D. A. Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. J. Cell Biol., *95:* 763–770, 1982.

48. Aquino, A., Hartman, K. D., Knode, M. C., Grant, S., Huang, K. P., Niu, C. H., and Glazer, R. I. Role of protein kinase C in phosphorylation of vinculin in Adriamycin-resistant HL-60 leukemia cells. Cancer Res., *48:* 3324–3329, 1988.

49. Laemmli, U. K. Cleavage of structural proteins during the assembly of the bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.