Induction of Protein Kinase C in Mouse Melanoma Cells by Retinoic Acid

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ABSTRACT

Retinoic acid inhibits the proliferation of B16 mouse melanoma cells. It also eliminates the ability of these cells to grow in soft agar. These biological actions of retinoic acid have been shown to be accompanied by an increase in the amount of cyclic AMP-dependent protein kinase and an induction of a new isozyme form (RII β). In this report we demonstrated that retinoic acid-treated B16 melanoma cells had large increases in protein kinase C activity.

This increased enzyme activity was accompanied by increases in both the number of phorbol dibutyrate binding sites and the amount of immunoreactive protein kinase C. Other treatments (melanocyte-stimulating hormone, serum deprivation) which inhibited the growth of these cells did not increase protein kinase C activity. When B16 melanoma cells were treated for a prolonged time (72 h) with phorbol dibutyrate, protein kinase C activity was barely detectable. Under these conditions, melanin production was inhibited and cell growth was accelerated. When retinoic acid was added together with phorbol dibutyrate, it prevented the growth stimulatory effect of the phorbol ester and increased protein kinase C activity. However, the absolute activity of the enzyme was still below that found in control cells and very much lower than in cells treated with retinoic acid alone. Together taken with our previous findings, we propose that the increase in protein kinase C might be part of a differentiation program induced by retinoic acid.

INTRODUCTION

PKC, a phospholipid- and Ca2+-dependent kinase that phosphorylates substrate proteins on serine and threonine residues, is found in almost all tissues, with the highest activity being observed in brain (1). PKC is activated by several growth factors, hormones, and neurotransmitters. It is also the major if not the only receptor for phorbol ester tumor promoters (2-4). Phorbol esters can stimulate proliferation of several cell types. In addition, PKC has been shown to phosphorylate the epidermal growth factor receptor (5), the insulin receptor (6), and the protein product of the ras oncogene (p21) (7). These data suggest that protein kinase C plays an important role in regulating cell proliferation.

RA has been shown to induce differentiation and/or inhibit the growth of many different types of tumor cells (8-12). In B16 mouse melanoma cells, RA specifically blocks progression through G1, as well as eliminating growth in soft agar (13). We have previously found that, in B16 melanoma, RA stimulates the activity of PKA and increases the amount of the R1 cyclic AMP-binding subunit (13, 14). A mutant of B16 melanoma cells having defective PKA did not have its growth inhibited by RA, implying that PKA may play a critical role in the pathway leading to RA-induced growth inhibition (13). In this report we demonstrate that RA treatment of B16 mouse melanoma cells leads to increases in both the activity and amount of PKC, a kinase which is of critical importance in control of growth.

MATERIALS AND METHODS

Cell Culture. B16 F1 cells were obtained from Dr. Fidler (M. D. Anderson Hospital and Tumor Clinic, Houston, TX) and were maintained as stock in Dulbecco's minimal essential medium that was supplemented with 2 mm L-glutamine, 2 mm sodium pyruvate, 50 units/ml penicillin G, 50 μg/ml streptomycin sulfate, and 10% newborn supplemented calf serum (Sterile Systems, Logan, UT). Every 2 months new cultures were initiated from frozen stock, in order to minimize the changes in cell phenotype that can occur with prolonged in vitro culture.

Treatment of Cells with Test Agents. B16 F1 cells were routinely seeded onto 100-mm tissue culture dishes (Falcon). The following day, cells were refed with growth medium (see above) with or without the following agents: MSH (Sigma), 5 μg/ml; all-trans-retinoic acid (Eastman Kodak), 10 μM; or PDB (Sigma), 1 μM. At the end of the incubation, replicate dishes were taken for determination of cell number by use of a hemocytometer. The remainder of the dishes were harvested for the various assays discussed below.

Protein Kinase C Assay. At selected time points, cells were harvested by aspirating off the growth medium, washing twice with cold phosphate-buffered saline, and scraping the cells into a small volume of lysis buffer [20 mM Tris (pH 7.5), 2 mM EDTA, 0.5 mM [ethylenebis(oxyethylenenitrito)]tetracetic acid, 5% Triton X-100, 1 mM dithiothreitol, 10 μg/ml aprotinin, and 5 mM phenylmethylsulfonyl fluoride]. The cell suspension was sonicated at power setting 3.5 for 20 s using the microtip of a Bronson sonifier. A soluble fraction was prepared by centrifuging the sonicated cells at 100,000 × g for 1 h. The supernatant was put into a 1-ml DEAE-cellulose column which had been preequilibrated in column buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM [ethylenebis(oxyethylenenitrito)]tetracetic acid, 1 mM dithiothreitol). The column was then washed with 15 ml of column buffer and eluted with 2 ml of column buffer that contained 100 mM NaCl (preliminary experiments showed that the majority of PKC eluted at this salt concentration). An aliquot of this fraction was then taken for protein analysis using the Bradford dye-binding assay (15) while another aliquot was taken for PKC assay. The standard reaction mixture for PKC analysis consisted of 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 1 mM CaCl2, 400 μg/ml histone type IIIS (Sigma), 50 μM ATP, with or without 16.5 μg phosphatidylserine/0.2 μg phorbol, 2 μCi [γ-32P]ATP (2-10 Ci/mmol; New England Nuclear), and 20 μg of sample protein in a total volume of 100 μl. The reaction was incubated at 30°C for 10 min, and then the entire reaction mixture was spotted onto Whatman P81 filter paper discs, dried, and then washed extensively in 0.2% phosphoric acid. After drying, the radioactive histone was quantitated by liquid scintillation counting. PKC specific activity is defined as the activity in the presence of phospholipid minus the activity in the absence of phospholipid per mg of protein.

Phorbol Dibutyrate Binding Assay. B16 cells were seeded onto 60-mm tissue culture dishes in growth medium and the following day were refed with or without 10 μM RA. At various times during the incubation with RA, the medium was aspirated and the cells were washed with Eagle's basal medium plus 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.2. They were incubated with 4 nM [3H]phorbol dibutyrate (0.1 μCi/ml; New England Nuclear) in the above medium (1.5 ml) with or without 5 μM unlabeled phorbol dibutyrate (Sigma). After a 4-h incubation, the medium was removed, the cells were washed twice with cold PBS and scraped into 0.5 ml of PBS and the radioactivity was determined.

Depletion of Protein Kinase C. Cells were seeded at 1.25 × 104/100-mm tissue culture dish in growth medium. The following day, one half

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3The abbreviations used are: PKC, protein kinase C; RA, retinoic acid; PBS, phosphate-buffered saline; TNS, 15 mM Tris-HCl, pH 7.4-0.85% NaCl; PDB, phorbol dibutyrate; DMSO, dimethyl sulfoxide; MSH, melanocyte-stimulating hormone; PKA, cyclic AMP-dependent protein kinase; TPA, tetradeacylphorbol acetate.
of the dishes were re-fed with medium containing 1 µM phorbol dibutyrate, while the remaining dishes (control) were re-fed with medium containing the solubilization vehicle (DMSO). After a 24-h incubation, replicate dishes were harvested for determination of cell number and protein kinase C activity, while the remainder were re-fed with medium containing the following agents: DMSO (control), phorbol dibutyrate (1 µM), retinoic acid (10 µM), or phorbol dibutyrate plus retinoic acid. These agents were incubated with the cells for 48 h, and then all plates were harvested for determination of cell number and protein kinase C activity by the methods outlined above.

Western Blot Analysis of Protein Kinase C. B16 cells treated with or without 10 µM retinoic acid for 48 h were harvested as described above for assay of protein kinase C enzyme activity. The 100,000 x g supernatant fractions were applied to a DEAE-cellulose column and the runoff plus a 150 mM NaCl eluate in column buffer were collected. These fractions were concentrated and desalted by filtration through Minicon (Amicon, Bedford, MA; M, 10,000 cutoff) units. Protein content was determined by the Bradford assay (15), and 100 µg of protein from each fraction were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were then electrophoretically transferred (300 mA, 4 h) onto nitrocellulose paper (Hybond C; Amersham). Two different sources of protein kinase C antibodies were used in this study. Using the monoclonal antibody MC-5 from Amersham, the blots were preincubated with 3% bovine serum albumin in PBS at 4°C overnight. They were then incubated with a 1:28 titer of the antibody in 0.1% bovine serum albumin/PBS overnight at 4°C. The following day, the antibody solution was removed and the blots were washed 8 times for 5 min with 0.1% Tween 20 in PBS. Antibody binding was visualized by incubating the blots with 3.5 µCi 125I-Protein A in 0.1% Tween 20/PBS overnight at 4°C. The blots were then washed with PBS/Tween until the radioactivity in the washes reached background levels. The second antibody used in this study was chicken anti-PKC, generously provided by Dr. Curtis Ashendel (Purdue University, West Lafayette, IN). Using this antibody, the blots were preincubated with TNS containing 5% Carnation nonfat dry milk, overnight, at 4°C. They were then incubated with a 1:500 titer of the antibody for 2 h at room temperature. The antibody solution was then removed and the blots washed 5 times for 5 min in TNS containing 1 ml/liter Tween 20. The blots were then treated for 15 min with TNS containing 5% Carnation nonfat dry milk and then incubated for 2 h at room temperature with rabbit anti-IgY (kindly provided by Dr. Ashendel) at a titer of 1:500. Following this incubation, the blots were washed 5 times for 5 min with TNS/Tween and treated for 15 min with TNS containing 5% Carnation nonfat dry milk, and then antibody binding was visualized by incubation with 125I-Protein A (3.5 µCi) overnight at 4°C. The blots were then washed with TNS/Tween until the radioactivity in the washes was reduced to background levels. Blots were wrapped in plastic wrap and exposed to X-ray film (Kodak X-AR) at -70°C for 48–72 h. For quantitation of the level of immunoreactive protein kinase C, the autoradiograms were scanned with an LKB laser densitometer (model Ultrascan XL) and the integrated areas under the peaks for each sample were compared.

RESULTS

Stimulation of Protein Kinase C Activity by Retinoic Acid. We have previously determined that MSH and RA arrest growth of B16 melanoma cells in G1 (12). Since protein kinase C has been implicated in regulating the growth of various cell types (16), we measured its activity in cells treated with the above agents and also in cells growth arrested by serum deprivation. Table 1 shows that all of the agents inhibited growth relative to the control cells; however, only RA stimulated PKC activity. The degree of stimulation varied between experiments but was generally in the range of 4–8-fold. The enzyme activity from both control and treated cultures was proportional to protein concentration and was predominantly (>95%) in the 100,000 x g soluble fraction (data not shown).

The relationship between the time of RA treatment and stimulation of PKC activity was examined. Fig. 1 shows that, by 24 h of treatment with 10 µM RA, B16 melanoma cells have a 4-fold higher specific activity of PKC, compared to untreated cells. The absolute specific activity of PKC from RA-treated cells increased 3-fold during the second 24 h of treatment; however, since the untreated cells had also higher PKC activity at this time point, the relative difference was still about 4-fold. It was technically difficult to measure time points beyond 48 h of treatment because the large amount of melanin produced by these cells became cytotoxic.

Phorbol Dibutyrate Binding Studies. In order to determine if the RA-induced increase in PKC activity was due to an increase in the number of PKC molecules, we performed two kinds of experiments. In the first experiment, we measured the specific binding of PDB to intact B16 mouse melanoma cells. Fig. 2A shows that with increasing time of RA treatment there is a corresponding increase in the specific binding of PDB. These data correlate with the time-dependent RA-induced increase in PKC activity. At 48 h of RA treatment, we compared the relative affinity of PDB binding to its receptor in control and treated cells (Fig. 2B). This Scatchard plot revealed both high and low affinity binding sites, similar to previous findings (17). However, we believe that the low affinity site may be due to nonspecific binding. The Kd for the high affinity receptor (PKC) in control and treated cells is approximately the same (20–35 nM); however, the number of receptor sites is 102 in control cells versus 3–4 x 107 in RA-treated cells.

Western Blot Analysis of Protein Kinase C Levels. The increase in the amount of PKC was verified by a second approach, in which we measured the steady state levels of PKC by the Western blot technique. B16 cells were incubated with and without 10 µM RA for 48 h. Equal protein aliquots from the crude 100,000 x g supernatant fractions and a 150 mM NaCl...
with PDB resulted in almost complete loss of PKC activity. With 10 μM phorbol dibutyrate had less than one-half the PKC eluate of a DEAE-cellulose column were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins were electrophoretically transferred to nitrocellulose and incubated with a monoclonal anti-PKC antibody as described in the text. Lanes 1 and 2, 20 μl and 100 μl, respectively, of purified rat brain PKC. Lanes 3 and 5, crude extract and DEAE-cellulose-purified fraction, respectively, from control cells. Lanes 4 and 6, crude extract and DEAE-cellulose-purified fraction, respectively, from retinoic acid-treated cells. This autoradiogram represents a 72-h exposure at −70°C of the blot in a cassette with double reflector screens.

Fig. 2. Increased phorbol dibutyrate binding in retinoic acid-treated B16 mouse melanoma cells. A, B16 cells were treated with or without 10 μM retinoic acid. At the indicated time points, triplicate plates of cells were incubated with [³H]phorbol dibutyrate and specific binding was determined as described in the text. OD. control; C, +10 μM retinoic acid. The bars above and below the data points represent the SE (three experiments). B, B16 cells were treated with or without 10 μM retinoic acid for 48 h. At this time, triplicate plates were incubated with [³H]phorbol dibutyrate in the absence or presence of the indicated concentrations of unlabeled phorbol dibutyrate and specific binding was determined as described in the text. The data are presented as a Scatchard plot (control Kₜ = 35 nM; RA Kₜ = 20 nM).

The Western blot in Fig. 3 illustrates two points. First, the antibody binds to several different concentrations of purified rat brain PKC (kindly provided by Dr. Curtis Ashendel, Purdue University, West Lafayette, IN) (Fig. 3, Lanes 1 and 2; Mr, 80,000 protein), and, second, absorption and elution of the crude 100,000 × g supernatant fractions from control and 48-h retinoic acid-treated cells on DEAE-cellulose columns result in enrichment of immunoreactive PKC (compare Fig. 3, Lanes 4 and 6). When Fig. 3, Lanes 5 and 6, were quantitated by densitometry scanning, there was a 20–25-fold greater amount of immunoreactive protein kinase C in samples from retinoic acid-treated cells. Using the more potent chicken anti-PKC antisera, we could detect an increase in immunoreactive PKC as early as 16 h of retinoic acid treatment (data not shown).

Depletion of Protein Kinase C. In order to determine if the RA-induced increase in PKC played a role in the growth response to RA, we took advantage of the fact that long term incubation of cells with phorbol esters results in a nearly complete loss of PKC activity (18, 19). B16 cells treated for 24 h with 1 μM phorbol dibutyrate had less than one-half the PKC activity of control cells, while an additional 48-h incubation with PDB resulted in almost complete loss of PKC activity (Table 2). PDB did not alter the growth of the cells during the first 24 h of treatment; however, in the subsequent 48-h incubation it stimulated growth by 3-fold. The treated cells also had a suppression of melanin production (cellular melanin concentration was 0.88 absorbance units in control cells versus 0.23 in PDB-treated cells, based on A₄₀₀/10⁶ cells). In cells that were treated with PDB for 24 h and then had the agent removed during the second incubation, there was no stimulation of cell proliferation, but we consistently found that PKC activity was higher than in control cells (Table 2). When RA was added to control cells during the second 48-h incubation, it inhibited growth and stimulated PKC activity, as seen in previous experiments (Table 1). In cells treated with PDB for 24 h and then incubated in the continued presence of PDB, but with the addition of RA for the second 48-h period, there was no PDB-induced stimulation of cell proliferation; however, RA did not inhibit proliferation relative to control cells. Under these conditions, RA stimulated cell proliferation to levels similar to control cells with PDB present.

Table 2 Effect of protein kinase C depletion on the ability of B16 melanoma cells to respond to retinoic acid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation 1 (24 h)</th>
<th>Incubation 2 (48 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell no. (×10⁶)</td>
<td>Protein kinase C activity (pmol ⁴P incorporated into histone/min/10⁶ cells or/mg cell protein)</td>
</tr>
<tr>
<td>Control</td>
<td>0.8</td>
<td>35 ± 1.1</td>
</tr>
<tr>
<td>1 μM PDB</td>
<td>0.7</td>
<td>17 ± 0.3</td>
</tr>
<tr>
<td>Control – Control</td>
<td>3.3</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>Control – RA (10 μM)</td>
<td>1.6</td>
<td>49 ± 98</td>
</tr>
<tr>
<td>PDB – control</td>
<td>2.4</td>
<td>122 ± 3.0</td>
</tr>
<tr>
<td>PDB – PDB</td>
<td>9.9</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>PDB – PDB + RA</td>
<td>3.8</td>
<td>25 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>± 0.2</td>
<td>± 3.0</td>
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<tr>
<td></td>
<td>± 13</td>
<td>± 3.9</td>
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Fig. 3. Western blot analysis of protein kinase C levels in control and 48-h retinoic acid-treated B16 mouse melanoma cells. B16 cells were seeded at 2 × 10⁶/100-mm dish in Dulbecco's medium plus 10% supplemented calf serum (Sterile Systems, Logan, UT). One day after seeding, the cultures were re-fed with either unsupplemented medium (control) or medium containing 1 μM PDB. Following a 24-h incubation (incubation 1), replicate dishes were harvested for determination of cell number and protein kinase C activity as described in "Materials and Methods." The remaining dishes were re-fed with medium containing the additives listed in the table and were incubated for an additional 48 h (incubation 2). At the end of this incubation, all dishes were harvested and cell number and protein kinase C activity were determined. Protein kinase C activity is expressed as pmol ⁴P incorporated into histone/min/10⁶ cells or/mg cell protein. The data are presented as the mean ± SE (three experiments).
ditions, RA still stimulated PKC activity relative to cells treated with PDB alone; however, the absolute activity was considerably less in control cells or cells treated with RA alone.

DISCUSSION

In this report we have shown that retinoic acid can induce a substantial increase in protein kinase C in B16 melanoma cells. This effect of retinoic acid is specific and not simply due to the growth state of the cells, since other agents which inhibited proliferation did not increase the activity of protein kinase C. The amount of time required before the increase in protein kinase C activity could be detected, together with the increases in the number of phorbol dibutyrate binding sites and the amount of immunoreactive protein kinase C (as deduced by Western blots), suggests that retinoic acid is increasing the number of protein kinase C molecules. Furthermore, retinoic acid treatment of the cells did not alter the affinity of protein kinase C for phorbol dibutyrate binding (Fig. 2B) or its affinity for calcium or phospholipid (data not shown). There is some discrepancy between the degree of increased protein kinase C measured by either enzyme activity or phorbol ester binding (4-8-fold) versus that quantitated by Western blots (20-25-fold). This could be explained if we were overestimating the PKC enzyme activity or phorbol ester binding activity in control cells. The amount of these two activities is very low in control cells, and a slight error could be magnified during the subsequent calculations. An alternative explanation is that not all of the immunoreactive PKC is enzymatically active. It does not appear to be degraded, since the $M_r$ of the immunoreactive material is 80,000, the same as authentic rat brain PKC. Further experiments will be needed to resolve this question.

It was previously shown that retinoic acid-induced differentiation of the human promyelocytic cell line HL-60 was accompanied by increased protein kinase C activity (20). Other agents, such as DMSO and vitamin D$_3$, which induced granulocytic differentiation of HL-60 also increased protein kinase C activity (21). In the case of vitamin D$_3$-induced differentiation of HL-60, there was also an increase in phorbol ester receptors (22). More recently, Makowske et al. (23), using antipeptide antibodies that specifically recognized the $\alpha$, $\beta$, and $\gamma$ isozymes of protein kinase C, showed that all three isozymes increased about 3-fold in abundance after a 96-h treatment of HL-60 cells with 1 $\mu$M retinoic acid. DMSO, which also induced differentiation, also increased the amount of all three isozymes, although there was a relatively greater increase in the $\alpha$ and $\beta$ forms of protein kinase C. In addition to HL-60, the human monoblastoid cell line U937 also exhibited increased protein kinase C activity when induced to differentiate by treatment with vitamin D$_3$ (24). It is not clear from these studies whether the increase in protein kinase C is a result of differentiation or the inhibition of cell proliferation which accompanies this process. We have previously shown (12) that both MSH and retinoic acid arrest B16 cell growth in the G$_1$ phase of the cell cycle. However, as shown in this report, only retinoic acid increases the relative amount of protein kinase C. Therefore, in these melanoma cells, the increase in protein kinase C cannot simply be a consequence of arrest in G$_1$.

In order to determine what role the increased amount of protein kinase C might play in the biological effects of retinoic acid, we took advantage of the fact that prolonged treatment of cells with phorbol esters results in depletion of protein kinase C (18, 19). Using phorbol dibutyrate, we found that a 24-h treatment markedly diminished protein kinase C activity, and a subsequent additional 48-h incubation with phorbol dibutyrate almost completely eliminated the activity of this enzyme. The B16 cells responded to prolonged phorbol dibutyrate treatment by an increased cell proliferation and a decrease in melanin production. Mufson et al. (25) also found that TPA inhibited melanin production in the C3 clone of B16 melanoma. However, they found that the phorbol ester did not alter growth. This may have been due to the fact that the concentration of TPA which they used ($10^{-8}$ to $10^{-7}$ M) was not sufficient to completely deplete the cell of protein kinase C. From our results, we cannot deduce whether the enhanced cell growth is directly due to the depletion of protein kinase C or a consequence of depressed melanin synthesis, the byproducts of which are cytotoxic. When retinoic acid was added to B16 cells in the continued presence of phorbol dibutyrate, most of the growth stimulation induced by the phorbol ester was eliminated. However, the ability of retinoic acid to inhibit growth relative to untreated controls was also negated. Thus, these two compounds have almost equal and opposing effects on B16 melanoma cell growth. Retinoic acid was also able to induce a 5-fold increase in PKC activity even in the continued presence of PDB. However, the absolute enzyme activity was still significantly below the untreated control level.

Phorbol esters have been shown to support the growth of normal melanocytes in culture by permitting preferential attachment of melanocytes from epidermal cell suspensions and then stimulating the attached melanocytes to grow (26). Since the phorbol esters were present continuously in the culture medium, it is likely that protein kinase C was depleted, a situation analogous to our treatment of B16 melanoma with phorbol dibutyrate. Also, a recent report has shown that, in C3H 10T$^{1/2}$ mouse fibroblasts transfected with the human Ha-ras oncoprotein, PKC is also down-modulated (27). Thus it may be that, in certain cell types, high levels of protein kinase C are incompatible with enhanced cell proliferation. In contrast to these results, it has been recently reported (28, 29) that NIH-3T3 cells or rat fibroblasts transfected with protein kinase C plasmids under the control of strong viral promoter/enhancer elements resulted in high expression of protein kinase C and disordered growth control. The transfected cells grew to a higher saturation density and formed small colonies in soft agar in the absence of TPA. These conflicting results suggest that different cell types respond differently to fluctuations in protein kinase C levels or that, at least in the case of retinoic acid, multiple biochemical pathways interact to result in growth inhibition.

It is not clear how retinoic acid increases protein kinase C levels. B16 cells contain retinoic acid-binding proteins,* but we have not determined whether this corresponds to the well-known cellular retinoic acid-binding protein (30) or the newly discovered retinoic acid receptor (31, 32). Since the retinoic acid receptor has the characteristics of a DNA-binding protein and belongs to the family of steroid receptor genes (31), it provides a potential pathway by which retinoic acid could directly influence the transcription of protein kinase C.

Finally, we are left with the question of what is the role of the retinoic acid-induced increase in protein kinase C. Since retinoic acid was still able to increase protein kinase C activity in melanoma cells which had been depleted of PKC by phorbol ester treatment, these experiments did not definitely settle whether this enzyme is involved in the retinoic acid-mediated growth inhibition. However, we would like to propose an alter-

*Niles and Traish, unpublished results.
native explanation for the increase in protein kinase C, i.e., that it is part of a differentiation program induced by retinoic acid. Melanocytes are derived from the neural crest during development and share many characteristics of nerve cells (e.g., presence of nerve growth factor receptors). Since the brain has the highest amount of protein kinase C of any organ, it is quite likely that mature melanocytes are also rich in this enzyme. Therefore, the retinoic acid-induced increase in protein kinase C in these melanoma cells could be indicative of differentiation to a more mature state. Examination of other melanocyte-specific markers in retinoic acid-treated B16 melanoma cells will be required to provide further evidence for this hypothesis.

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