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Regulation of Retinoic Acid Receptor α by Protein Kinase C in B16 Mouse Melanoma Cells*

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We have previously found that retinoic acid stimulates the expression of protein kinase Cα (PKC) in B16 mouse melanoma cells. Because it has been reported that PKC can phosphorylate retinoic acid receptor (RAR) and alter its function, we determined whether changes in the level and/or activity of PKC could affect the expression or function of the RAR in B16 melanoma. Using *in vivo* phosphorylation and band shift techniques, we could not demonstrate that altering PKC activity and/or protein level changed the *in vivo* phosphorylation of RARα. However activation of PKC resulted in increased RARα protein. Increased receptor protein correlated with a phorbol dibutyrate-stimulated increase in receptor activation function-2 (AF-2)-dependent transcriptional activity. Use of enzyme inhibitors and dominant-negative PKCs indicated that enzyme activity was required for elevation in the RARα. The PKC-mediated increase in RARα was due to a 2.5-fold increase in the half-life of this protein. In contrast, the down-regulation of PKC diminished RARα protein half-life and markedly inhibited AF-2-dependent transcriptional activity. The down-regulation of PKC also inhibited the binding of RAR to a retinoic acid response element and the retinoic acid induction of RARβ expression. These findings suggest that PKC can influence retinoic acid signaling by altering the stability of RAR protein without directly phosphorylating this receptor.

All *trans*-retinoic acid (RA)[1] is the major biologically active form of vitamin A. This vitamin is important for normal growth and development, especially during embryogenesis where a three-dimensional gradient of RA determines pattern formation (1). It is also an important regulator of reproduction (2), differentiation (3), and apoptosis (4). Additionally, RA has been found to inhibit the growth of malignant cells (5, 6) and/or to induce reversion from the malignant to the normal phenotype (7). Most, if not all, of all these effects are mediated though retinoid nuclear receptors. There are two families of retinoid nuclear receptors: RARs and RXRs. Each family of nuclear retinoic acid receptors consists of α, β, and γ subtypes (8–10). These nuclear receptors are ligand-dependent transcriptional regulators, with RXR being activated by 9-*cis*-RA, whereas RAR are activated by either all-*trans*-RA or 9-*cis*-RA (11–13). RXR can form homodimers with a number of other type II nuclear receptors, including RAR (14, 15). In contrast, RAR only forms heterodimers with RXR (16). Following ligand binding, RXR·RAR heterodimers bind to the promoter of target genes and stimulate their transcription (17). In B16 mouse melanoma cells RARα and RAR γ are constitutively expressed, whereas RARα expression can be induced by incubation of cells with RA (18).

Nuclear retinoic acid receptors are phosphoproteins. RARα is phosphorylated *in vivo* by cyclic AMP-dependent protein kinase (PKA) (19) and cyclin-activating kinase (CAK) (20). Phosphorylation of RARα by PKA in F9 cells is required for RA-induced parietal endoderm differentiation (21). In contrast, proline-directed phosphorylation sites (such as those phosphorylated by CAK) in RARγ were required for RA induction of primitive endoderm differentiation (22). The PKA phosphorylation site in RARγ was dispensable for this biological activity of RA (21).

Protein kinase C (PKC) is a serine/threonine protein kinase and plays a role in the regulation of growth, differentiation, and other cellular functions (23). The PKC family includes at least 11 different enzymes that are classified into three families based on requirement for phospholipids, diacylglycerol, or Ca^{2+} for their activation (24, 25). Following activation, PKC is translocated to different cellular compartments where it phosphorylates substrate proteins. In addition, PKC has been shown to transverse the nuclear membrane and phosphorylate targets in the nucleus (26). The transcription complex activating protein 1 is activated by PKC (27), which provides a means for PKC to influence gene expression.

It has been proposed that there is an interaction between PKC and retinoic acid receptors. PKC has been shown to phosphorylate RAR *in vitro* (28, 29). On the other hand, one group has reported that RA binds to PKC in a specific manner and inactivates its enzymatic activity (30). We have previously reported that treatment of B16 melanoma cells with RA increases the amount of PKCα mRNA and protein (31). In light of the purported interaction between PKC and RAR, we investigated whether the increased amount of PKCα in B16 cells might phosphorylate the RAR and alter its function. We found, in B16 mouse melanoma cells, that RARα is not a target for PKC-directed phosphorylation. However, we discovered that activation of PKC in these cells resulted in an increase in RARα protein. The increase in RARα was due to an increase in the half-life of this protein, which correlated with an increase in AF-2 activity. In contrast, down-regulation of PKC through

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[1] The abbreviations used are: RA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; CAK, cyclin-activating kinase; PKC, protein kinase C; AF-2, ligand-dependent transcriptional activation; PDB, phorbol 12,13-dibutyrate; PKA, cyclic AMP-dependent protein kinase; DMEM, Dulbecco's modified Eagle's medium; RARE, retinoic acid response element; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; mRAR, mouse retinoic acid receptor.

prolonged phorbol dibutyrate (PDB) treatment led to a large decrease in AF-2 activity, which cannot be totally accounted for by diminished RAR stability. Our studies suggest that there exists a significant cross-talk between the PKC and RAR signal transduction pathways.

EXPERIMENTAL PROCEDURES

Cell Culture—B16 mouse melanoma cells were grown in DMEM (Invitrogen, Rockville, MD) supplemented with 10% bovine calf serum (HyClone Laboratories, Logan, UT) at 37 °C, in a 5% CO_2/95% air, humidified atmosphere. Cells were treated for the periods described in each experiment with 10 μM phorbol 12,13-dibutyrate (PDB) (Sigma Chemical Co., St. Louis, MO) or 10 μM all-*trans*-RA (Fluka Chemical Corp., Ronkonkoma, NY). All manipulations involving RA were conducted under subdued lighting or yellow light to prevent photo-oxidation.

Cloning of mRARα into a Histidine-tagged Expression Vector—A mRARα cDNA fragment encoding the complete open reading frame, except for the last three C-terminal amino acids was produced by PCR using the following primers: 5′-GGACTGGCCAGCAACAGC-3′ (upstream primer) and 5′-GGCCGGGCTGCTTCTGTT-3′ (downstream primer) using the Advantage cDNA PCR kit (CLONTECH, Palo Alto, CA). The template for this reaction was mRARα in pSG5 that was kindly provided by Dr. Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Celluaire, CNRS/INSERM, Strasbourg, France). PCR was conducted by incubating at 94 °C for 1 min followed by 20 cycles at 94 °C for 30 s and 68 °C for 3 min, and ending with incubation at 68 °C for 3 min. Following authentication of the PCR product by DNA sequencing, it was cloned into the pcDNA3.1/V5/His-TOPO plasmid (Invitrogen, Carlsbad, CA) producing RARα-His. *In vitro* transcription/translation of this construct resulted in a protein that was about 5 kDa larger than wild type RARα. Histidine-tagged RARα had the same ligand binding and DNA binding properties as wild type RARα (data not shown).

Kinase-inactive PKCα Constructs—Two plasmids expressing enzymatically inactive PKCα were gifts from Dr. S. Ohno, Yokohama City University School of Medicine, Japan. One of these constructs (PKCα-Kn-SM) has a single K368R mutation in the ATP binding region. The other construct (PKCα-Kn-TM) has the same K368R mutation in addition to two amino acid mutations (R22A and A25E) in the pseudosubstrate region. Both PKCα mutants expressed from these constructs have no kinase activity. PKCα-Kn-SM is thought to be in a "closed" conformation, because it lacks the ability to autophosphorylate to achieve the mature conformation. PKC-Kn-TM, in contrast, is thought to have an "open" conformation due to the mutation in the pseudosubstrate region. B16 clones expressing these mutant PKCα proteins were used to examine the requirement of PKC enzyme activity for its effect on RAR stability.

Transient Transfections—RARα-His, pSG5 RARα, β, or γ (gifts from Dr. Chambon), RARE-tk-luciferase (gift from Dr. Ozato, Laboratory of Molecular Growth Regulation, National Institutes of Health, Bethesda, MD), and pSV-β-gal plasmids were transiently transfected into B16 cells using LipofectAMINE Plus (Invitrogen, Rockville, MD) according to the manufacturer's recommended protocol. For reporter gene assays, transfected cells were treated with PDB and/or RA for the indicated times, and 48 h after transfection cells were harvested and assayed for luciferase or β-galactosidase activity using kits from Promega (Madison, WI).

Western Blotting—Cells were lysed in buffer A (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 10% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, and 3.28 μg/ml PMSF) by three consecutive 10-s sonications with a Tekmar sonic disrupter at power setting 60. Proteins were separated by SDS-PAGE (5% stacking gel; 10% separating gel) and transferred to nitrocellulose (Micron Separation, Inc., Westborough, MA) using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA). The membranes were incubated with 10% nonfat dry milk overnight at room temperature. The membrane was then washed 3× with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20) for 5 min each, at room temperature and then probed with a 1:5000 dilution of anti-V5 monoclonal antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Subsequently, the membrane was washed as described above and further incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham Biosciences, Piscataway, NJ) at room temperature for 1 h. The membrane was washed three times with TBST and developed using the ECL chemiluminescence kit (Amersham Biosciences). Intensity of the RARα-His immunoreactive bands was quantified using a Molecular Dynamics densitometer (Sunnyvale, CA).

λ-Phosphatase Treatment of RARα-His—B16 cells were transiently transfected with the plasmid encoding the RARα-His protein as described above, harvested in buffer A and disrupted by sonication. 50 μg of protein extract was treated with 100 units of λ-phosphatase (New England BioLabs, Beverly, MA) in 50 mM Tris-HCl, pH 7.8, 0.1 mM EDTA, 5 mM dithiothreitol, 0.01% Brij 35, 2 mM $MnCl_2$ in a total volume of 20 μl for different time periods at 30 °C.

In Vivo Phosphorylation of RARα—B16 cells were transfected with the RARα-His expression vector. Twenty-four hours after transfection, medium was changed and cells were incubated overnight in phosphate-free DMEM (Invitrogen, Carlsbad, CA). Cells were then incubated for 4 h in the same medium in the presence of 250 μCi of [^{32}P]orthophosphate (Amersham Biosciences) and 100 μM orthovanadate (Sigma Chemical Co., St. Louis, MO). At the end of this incubation, cells were washed and harvested in cold PBS. RARα was purified by immunoprecipitation as described below and separated by SDS-PAGE on a 7.5% gel. The gel was dried and then exposed to x-ray film for 72 h.

Immunoprecipitation and Determination of RARα-His Protein Half-life—B16 cells were transfected with the RARα-His construct as described previously. At 44 h post-transfection, media was removed and replaced with methionine-free DMEM (Invitrogen, Rockville, MD) for 1 h. This medium was then replace with fresh methionine-free medium to which 100 μCi/ml [^{35}S]methionine (PerkinElmer Life Sciences, Boston, MA) was added, and the cells were incubated for an additional 2 h. At different times of experimental treatment, medium was removed and cells were washed with PBS and harvested in 0.9 ml of radioimmune precipitation buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 100 μg/ml PMSF, 30 μg of aprotinin, 1 mM sodium orthovanadate in PBS). Cells were disrupted by repeated aspiration through a 21-gauge needle. The cell lysate was centrifuged at 10,000 × *g* for 10 min at 4 °C, and the supernatant was precleared by the addition of 1 μg of mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) together with 20 μl of protein A-agarose (Santa Cruz Biotechnology) for 30 min at 4 °C. The protein A-agarose was removed by centrifugation at 2500 × *g* for 5 min at 4 °C, 0.7 μg of anti-V5 epitope antibody was then added, and the mixture was incubated for 1 h at 4 °C. Immune complexes were collected by the addition of 20 μl of protein A-agarose for 1 h at 4 °C followed by centrifugation at 2500 × *g* for 5 min at 4 °C. The immunoprecipitate was washed three times by resuspension in 1 ml of radioimmune precipitation buffer followed by centrifugation as described previously. The pellet was resuspended in 30 μl of SDS samples buffer, and 25 μl was loaded onto a 5% stacking/10% separating SDS gel for protein separation by electrophoresis. The gel was then dried using a Model 583 gel drier (Bio-Rad, Hercules, CA), and the RARα-His protein was detected by autoradiography. The amount of RARα-His was determined by densitometry.

Northern Blotting—RNA was isolated by a single-step method as described previously (32). The RNA was then fractionated on 1% agarose containing formaldehyde and transferred to Hybond N nylon membranes (Amersham Biosciences, Chicago, IL) by downward alkaline blotting. The transferred RNA was cross-linked to the membrane by UV light. The membrane was prehybridized for 1 h in 6× SSC and 2% SDS. ^{32}P-Labeled cDNA probes (1×10^6 dpm/ml) were then incubated with the membranes in fresh hybridization solution for 20 h. Blots were washed three times for 15 min each in 1× SSC plus 0.1% SDS, 0.5× SSC plus 0.1% SDS, and 0.2× SSC plus 0.1% SDS, respectively. The blots were exposed to Kodak XAR film in cassettes at −70 °C for 2–5 days. All the cDNA probes were labeled using the "prime-a-gene" labeling system from Promega (Madison, WI) plus 0.25 mCi of [α-^{32}P]dCTP. The relative amount of the different RNA species was quantitated by imaging the autoradiogram with a Molecular Dynamics laser densitometer, making sure that the signals were within the linear range of the instrument. The data are expressed as the ratio of the specific mRNA to the internal control, glyceraldehyde-3-phosphate dehydrogenase.

Electrophoretic Mobility Shift Assay—Two 33-bp DNA oligonucleotides were annealed to form a double-stranded oligonucleotide, which contained a retinoic acid response element corresponding to that found within the human RARβ gene promoter (33). This βRARE was labeled with [α-^{32}P]dCTP (3000 Ci/mmol) by end-filling of the 5′-protruding termini with the Klenow fragment of DNA polymerase I. The labeled βRARE was purified using a Qiagen Tip-20, precipitated with 10 mg of glycogen carrier in ethanol:acetone (1:3, v/v) at −80 °C, and resuspended in double distilled H_2O.

Gel shift reactions typically contained B16 nuclear extract (25 μg) or Sf9 cell extracts from recombinant hRARα1 baculovirus-infected cells (1 μg), 10% (v/v) glycerol, 10× reaction buffer (0.2 M HEPES, pH 7.9, 0.6 M KCl, 0.01 M Na_2EDTA, 0.1 M monothioglycerol, and 0.01 M PMSF), 2.5 μg of poly(dI-dC), and 2×10^4 cpm of [α-^{32}P]βRARE in a 20-ml total volume. The reactions were incubated for 20 min at 23–24 °C and

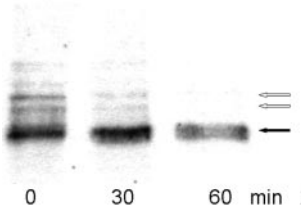

FIG. 1. **Ectopically expressed His_6-RARα is phosphorylated.** B16 cells were transfected with the His_6-RARα plasmid. At 24 h following transfection, cells were extracted and incubated with λ-phosphatase as described in the text. Equal amounts of control or treated extract proteins were separated by SDS-PAGE in a manner that allowed maximal separation of phosphorylated forms of RARα (receptor protein was allowed to migrate to the bottom of a 10% SDS gel). The His_6-RARα was detected by Western blots using an antibody against the V5-epitope found in fusion protein. The *open arrows* indicate the phosphorylated species of RARα.

immediately resolved at 20 mA at 23 °C in a 5% non-denaturing gel (60:1, acrylamide:bis) in 0.5× TBE (0.045 M Tris borate, pH 8.0, 0.001 M Na_2EDTA). Dried gels were exposed to film between intensifying screens at −80 °C.

All experiments described under "Results" were repeated three separate times with similar qualitative results.

RESULTS

RARα Ectopically Expressed in B16 Melanoma Cells Is Phosphorylated—Expression of endogenous RAR in B16 cells is very low and below the detection limit of Western blotting/immunoprecipitation techniques. Thus, for the studies reported here, we used recombinant histidine-tagged RARα transiently transfected and expressed in B16 cells. Histidine-tagged RARα protein had the same ligand binding and DNA binding properties as wild type RARα (data not shown). Expression of the recombinant RARα was confirmed by Western blotting, using an antibody directed against the histidine tag or an antibody to the V5 epitope, which is also part of the tagged protein. Three immunoreactive RARα bands were most often observed in extracts of transfected B16 cells, corresponding to 50–54 kDa. When these extracts were treated with λ-protein phosphatase for different periods of time, the two RARα bands of higher molecular mass collapsed into the lowest molecular mass band (Fig. 1) indicating that the His_6-RARα protein is phosphorylated in B16 cells. Elsewhere, in data presented, multiple bands of RARα may not be apparent. This is due to different gel running conditions and signal detection conditions required to display these multiple bands.

Activation and Down-regulation of PKC Does Not Alter the Phosphorylation of RARα in B16 Mouse Melanoma Cells—We have previously shown that incubation of B16 cells with phorbol dibutyrate for 1 or 24 h activates or down-regulates PKCα, respectively (34). Therefore, we used these phorbol dibutyrate treatment times to determine whether activation/down-regulation of PKC altered the phosphorylation of RARα. Fig. 2*A* shows that treatment of B16 cells with phorbol dibutyrate did not change the number of the RARα-immunoreactive bands (although the amount of RARα protein was higher in the 1-h phorbol dibutyrate-treated cells, see below). Also, the ratio of the relative amount of the different bands within each treatment group was not altered. In addition, radioactive labeling of intact cells with $^{32}P_i$-orthophosphate did not reveal any change in the amount of incorporation of the label into His_6-RARα in short or long term phorbol dibutyrate-treated cells when the signals were corrected for amount of receptor protein (Fig. 2*B*).

Increase in PKC Activity Results in Higher RARα Protein Levels in B16 Cells—Even though changes in PKC activity/expression did not alter the phosphorylation of RARα, an increase in PKC activity did result in a consistent 2-fold elevation in RARα protein levels (Fig. 2*A*). In contrast, down-regulation

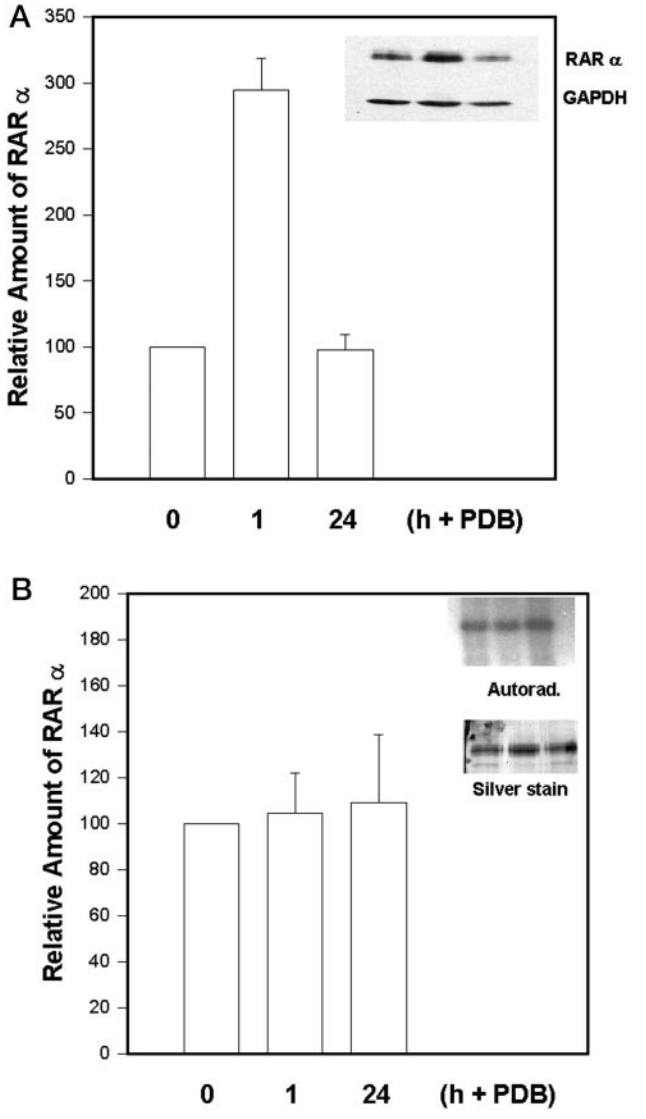

FIG. 2. **Effect of PKC activation or down-regulation on RARα phosphorylation.** B16 cells were transfected with the His_6-RARα plasmid. At 24 h post-transfection they were treated with 10 μM phorbol dibutyrate for 24 h (PKC down-regulation) or for 1 h (PKC activation) prior to harvesting. His_6-RARα was determined by Western blotting using the antibody against the V5 epitope (*A*). B16 cells transiently transfected with the RARα construct were incubated in the presence of [^{32}P]orthophosphate for 4 h and 10 μM phorbol dibutyrate for 1 or 24 h. RARα was then isolated by immunoprecipitation and separated on 7.5% SDS gels. The radioactive bands were detected by autoradiography, and the relative amount of phosphorylated RARα (*B*) was determined by densitometry of both the autoradiogram and silver-stained gels for estimates of total RARα protein (*insets*). The data are presented as the mean relative level of phosphorylation, corrected for the amount of total RARα of three separate experiments. The *error bars* represent the S.E. of the three experiments.

of PKC activity did not markedly change the level of RARα protein when compared with the control.

To explore this phorbol dibutyrate-induced change in RARα protein in more detail, B16 cells were incubated with phorbol dibutyrate for different periods of time and the amount of RARα protein was determined by Western blotting. A delay of 30 min was observed before a change in RARα protein levels could be detected. RARα protein levels reached a maximum, of about 3-fold over the control level, at 10 h following phorbol dibutyrate addition (Fig. 3*A*). Subsequently, RARα protein levels declined and reached control levels 24 h following addition of phorbol dibutyrate. These levels remained unchanged for the

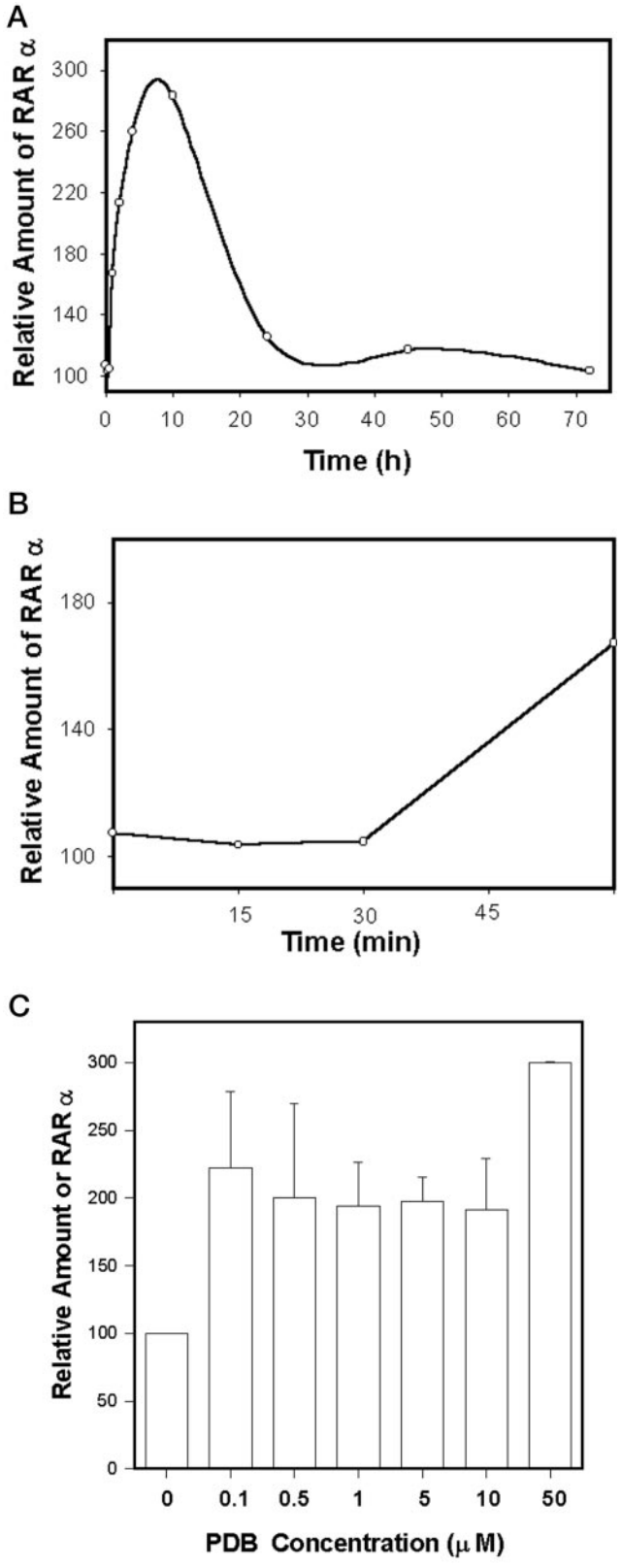

FIG. 3. **Effect of time and concentration of phorbol dibutyrate treatment on the amount of His_6-RARα.** Cells were transfected with the His_6-RARα plasmid. Transfected cells were treated with 10 μM phorbol dibutyrate for the times indicated (*A*) or for 1 h with the indicated concentrations of phorbol dibutyrate (*C*). Cells were then harvested, and the relative amount of His_6-RARα was determined by densitometry of the Western blots. The *panel* in *B* shows the amount of His_6-RARα at time points of 15 min, 30 min, and 1 h after adding 10 μM phorbol dibutyrate to the transfected cells. The data are presented as the mean relative amounts of His_6-RARα ± S.E. (*error bars*) of three separate experiments.

remainder of the 72-h treatment period.

The change in RARα protein level as a function of phorbol dibutyrate concentration was also analyzed. A concentration of 0.1 μM phorbol dibutyrate was able to increase RARα protein levels by 2-fold following a 1-h treatment (Fig. 3*C*). Further increases in phorbol dibutyrate concentration up to 50 μM did not substantially increase the level of RARα protein. A 50 μM concentration of phorbol dibutyrate increased the RARα protein level by an additional 50%.

PKC Activity Is Required for the Increase in RARα Protein Levels in B16 Cells—We investigated whether PKC enzyme activity was required for the observed increase in RARα protein by using both a chemical enzyme inhibitor and dominant-negative mutants of PKCα, which lack enzymatic activity. Bisindolylmaleimide, a selective PKC enzyme inhibitor, completely abolished the phorbol dibutyrate-induced increase in RARα protein (Fig. 4*A*). Clones of B16 cells stably expressing two different types of PKCα dominant-negative mutants were tested for the ability of phorbol dibutyrate to increase the amount of ectopically expressed RARα. Both of these clones have markedly less PKC-specific enzyme activity than wild type B16 cells (Table I). Short or long term phorbol dibutyrate treatment was not able to significantly change RARα protein levels in these clones of B16 cells further demonstrating the requirement for PKC enzyme activity to increase the amount of RARα protein (Fig. 4*B*).

PKC Regulates Stability of the RARα Protein in B16 Cells—The PKC enzyme-dependent increase in RARα protein could be the result of either increased mRNA or increased stability of the protein. Because we observed the increase in RARα protein within 1 h of addition of PDB to the cells, we considered an effect at the RNA level unlikely, thus we examined the effect of PKC activation on the stability of RARα protein. The half-life of ectopically expressed RARα in B16 cells was determined to be 4 h. When B16 cells were treated with PDB for 1 h, the RARα protein half-life increased to somewhat greater than 8 h (Fig. 5). In contrast, when PKC was down-regulated by a 24-h treatment with phorbol dibutyrate, the half-life of RARα decreased slightly to ~3 h (Fig. 5).

Altering PKC Activity Changes the Transcriptional Activation Function of the RAR—To further study the effect of PKC on RARs, RAR-dependent transcriptional activity was measured in an RARE reporter assay using thymidine kinase promoter/luciferase reporter plasmid. In response to PKC activation RAR-dependent transcriptional activity was increased ~2.8-fold when compared with the control (untreated) cells. On the other hand, down-regulation of PKC resulted in a 2-fold decrease in the transcriptional activity (Fig. 6*A*). Similar effects were observed with ectopically expressed RARs (Fig. 6*B*). One-hour PDB treatment resulted in an increase in RA-stimulated reporter gene activity in all of the RARs. In contrast, the down-regulation of PKC resulted in a large reduction of RA-stimulated reporter gene activity in all of the ectopically expressed RARs.

Lower RAR-dependent Transcriptional Activity Is a Result of the Less RAR Being Bound to the DNA—A gel shift assay was conducted using nuclear extracts from the cells treated with phorbol dibutyrate for 1 and 24 h. The cells were also treated with 10 μM RA for 24 h. The DNA·protein complex contains RARs, because it was possible to supershift the complex using the RAR antibody. Pre-treatment of the cells with RA increased the amount of the shifted complex (*lane 3*, Fig. 7). When nuclear extracts from B16 cells treated for 24 h with phorbol dibutyrate were used in gel shift assays, a decrease in the amount of the shifted complex was observed (*lane 5 versus lane 2*, Fig. 7). A small increase in the amount of RAR bound to DNA

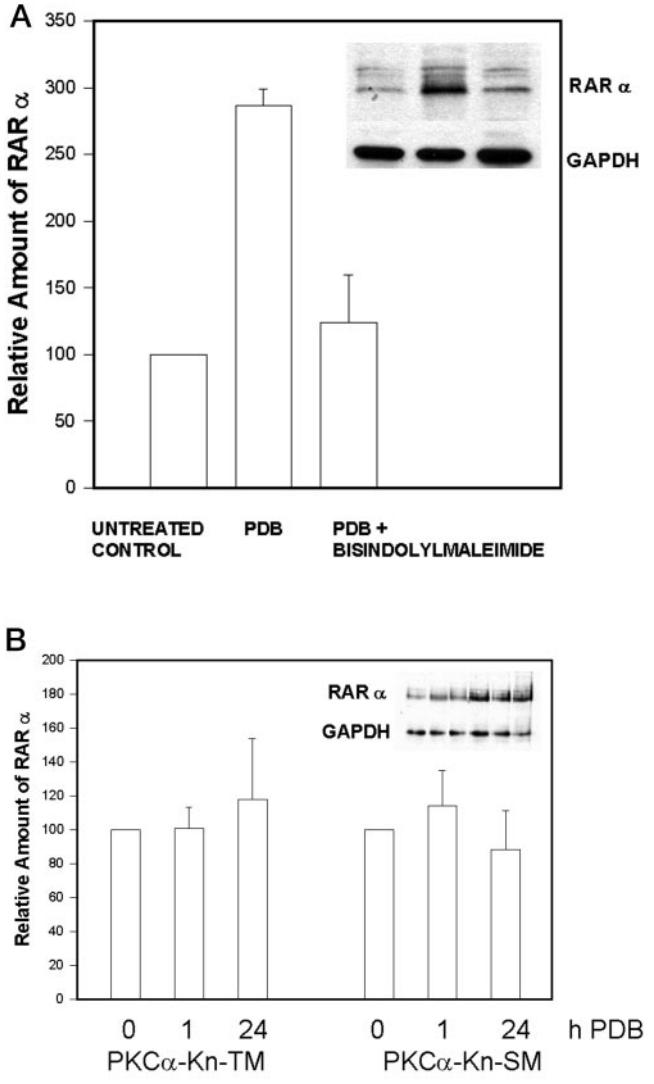

FIG. 4. **PKC enzyme activity is required for phorbol dibutyrate to increase the amount of His_6-RARα protein.** *A*, transfected cells were treated with 10 μM phorbol dibutyrate alone for 1 h or with 10 μM phorbol dibutyrate plus 2.5 μM bisindolylmaleimide (PKC enzyme inhibitor) for 1 h. Relative amounts of His_6-RARα were detected on Western blots using the V5 epitope antibody (the *inset* is from a representative experiment). The data are expressed as the mean of the relative amount of RARα (normalized for the amount of glyceraldehyde-3-phosphate dehydrogenase) ± S.E. (*error bars*) from three separate experiments. *B*, B16 clones KR23 (Lys to Arg mutation at amino acid 368 within the ATP-binding domain) and RE21 (Arg to Ala at amino acid 22; Ala to Glu at amino acid 25 within the pseudosubstrate region; and Lys to Arg mutation at amino acid 368 in the ATP-binding domain) were transfected with the His_6-RARα plasmid. 24 h post-transfection they were treated with phorbol dibutyrate as indicated. The relative amount of His_6-RARα was detected by Western blots using the V5 epitope antibody (*inset* from a representative experiment). The data are expressed as the mean of the relative amount of RARα (normalized for the amount of glyceraldehyde-3-phosphate dehydrogenase) ± S.E. (*error bars*) from three separate experiments.

TABLE I
PKC enzyme activity in wild type and PKCα mutant-expressing B16 clones

Cells were lyzed via sonication on ice with 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 5% Triton X-100, 0.5 mM PMSF, and 10 μg/ml aprotinin. The cell lyzate was centrifuged at 12,000 × *g* for 15 min. The supernatant was loaded onto a DEAE-cellulose anion exchange column (Cellex-D, Bio-Rad), previously equilibrated with column buffer (20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol). The column was eluted with several column volumes of column buffer containing 100 mM NaCl. Protein in the eluate was concentrated in a Centricon-30 microconcentrator (Amicon). Protein concentrations were then determine by the Bradford method, and samples were diluted to equal protein amounts prior to assay with the PKC enzyme assay kit from Amersham Biosciences. This system uses synthetic, PKC-specific substrate peptides, which become phosphorylated with the radiolabeled phosphate group from [γ-^{32}P]ATP. PKC-specific enzyme activity was determined by activity in the absence and presence of a phosphollipid, calcium/phorbol dibutyrate mixture. Radiolabeled peptide was separated from unincorporated [^{32}P]ATP by use of high affinity blotting paper. Enzyme activity was calculated as pmol of ^{32}P incorporated into the peptide/min/mg of protein. KR 23 is a G418-resistant clone derived from transfection of B16 cells with a plasmid encoding PKCα with a mutation in the ATP-binding site. RE 2.1 is a G418-resistant clone derived from transfection of B16 cells with a plasmid encoding PKCα with mutation in both the pseudo-substrate region and in the ATP-binding site.

Cell type	Enzyme activity
	pmol of ^{32}P/min/mg protein
B16 wild type cells	29.05
Clone KR 23	3.73
Clone RE 2.1	0.03

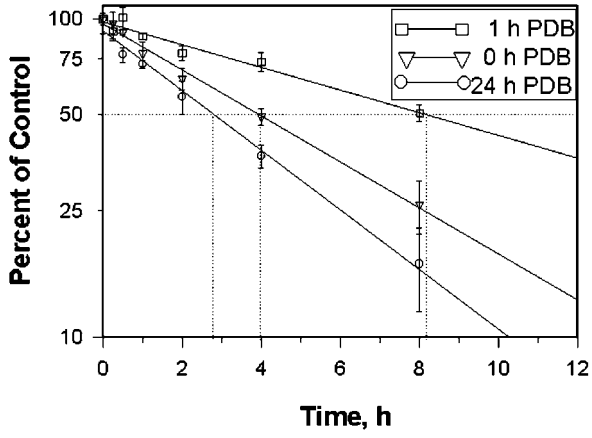

FIG. 5. **Effect of PKC activation/down-regulation on the half-life of His_6-RARα protein.** At 44 h post-transfection cells were pulsed with 100 μCi of [^{35}S]methionine for 2 h. The cells were then incubated for the indicated times in non-radioactive medium. At each time point, cells were lysed and the radiolabeled His_6-RARα was immunoprecipitated with antibody to V5 epitope followed by capture of the immune complexes on protein A-agarose beads. The immune complexes were then separated by SDS-PAGE, and the amount of His_6-RARα was determined by densitometry of the autoradiogram. Cells were treated with 10 μM phorbol dibutyrate during the last hour of the pulse period or treated with 10 μM phorbol dibutyrate for 24 h prior to the pulse period. The data are plotted as the percentage of the mean radioactive signal from three separate experiments ± S.E. (*error bars*) for His_6-RARα measured at the time cells were placed into to non-radioactive medium.

was observed with nuclear extracts from cells in which PKC activity was elevated (*lane 4*, Fig. 7).

PKC Down-regulation Inhibits the Retinoic Acid Induction of RARα mRNA in the B16 Mouse Melanoma Cells—RARα is not expressed under normal conditions in B16 mouse melanoma cells. However, it contains a retinoic acid response element (RARE), and therefore its expression can be induced by incubation with retinoic acid. Treatment of B16 cells with 10 μM RA for 4 h results in a 20- to 25-fold induction in RARα mRNA (18). When PKC activity in B16 cells was manipulated by short and long term phorbol ester treatment, it was observed that long term treatment, which down-regulates PKC activity, inhibited the induction of RARα mRNA by 80% (Fig. 8). Short term treatment of cells with phorbol dibutyrate did not change the amount of RARβ mRNA induced by retinoic acid.

DISCUSSION

We have previously found that treatment of B16 mouse melanoma cells with retinoic acid induces a 6- to 8-fold increase in PKCα and that this increase appears to be required for growth

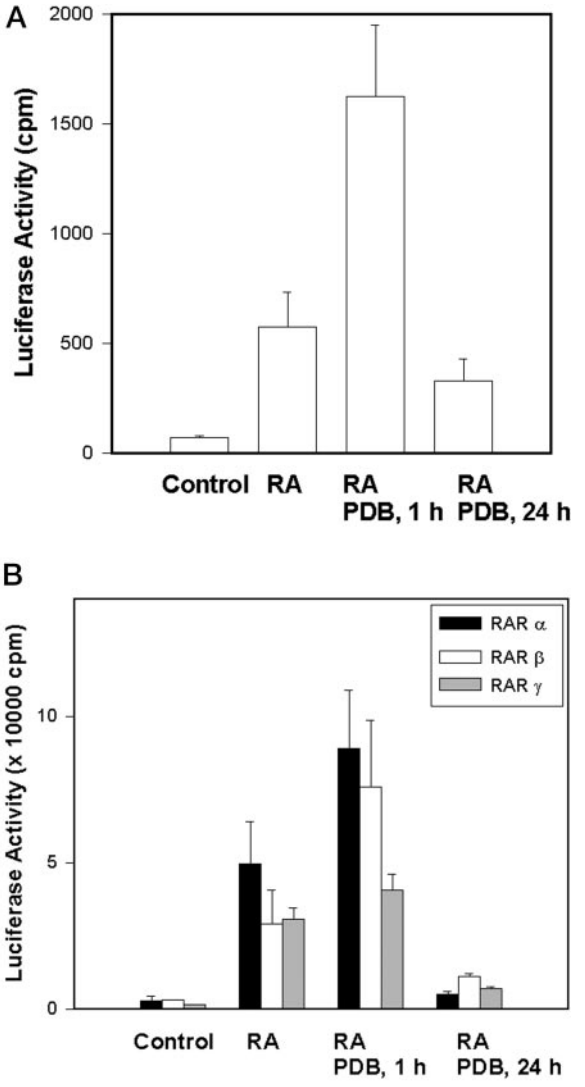

FIG. 6. **Effect of PKC activation/down-regulation on RAR transcriptional transactivation activity.** B16 cells were transfected with an RARE-tk-luciferase reporter plasmid without (*A*) or with (*B*) co-transfection of pSG5-RARα, β, or γ expression plasmids. 24 h post-transfection, cells were treated with 10 μM retinoic acid alone or with 10 μM phorbol dibutyrate (PKC down-regulation). For activation of PKC, cells were treated with 10 μM phorbol dibutyrate during the final 2 h of incubation with retinoic acid. *A*, reporter gene activity in cells containing only the endogenous RAR. *B*, reporter gene activity in cells ectopically expressing RARα, β, or γ.

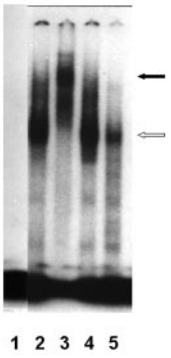

FIG. 7. **Down-regulation of PKC decreases RAR binding to RARE.** B16 cells were treated with 10 μM retinoic acid for 48 h in the absence or presence of 10 μM phorbol dibutyrate also for 48 h (PKC down-regulation) or for the last hour of retinoid treatment (PKC activation). Cells were then harvested, and nuclear extracts were prepared and incubated with a ^{32}P-labeled DNA oligonucleotide corresponding to a consensus DR-5 element. Complexes were separated on polyacrylamide gels, and bands were detected by autoradiography. *Lane 1*, DNA probe only; *lane 2*, DNA plus nuclear extract. The *open arrow* indicates the RAR·DNA complex; *lane 3*, DNA plus nuclear extract plus RAR antibody. The *closed arrow* indicates the "super-shifted" complex. *Lane 4*, DNA plus nuclear extract from cells treated with phorbol dibutyrate for 2 h; *lane 5*, DNA plus nuclear extract from cells treated with phorbol dibutyrate for 24 h.

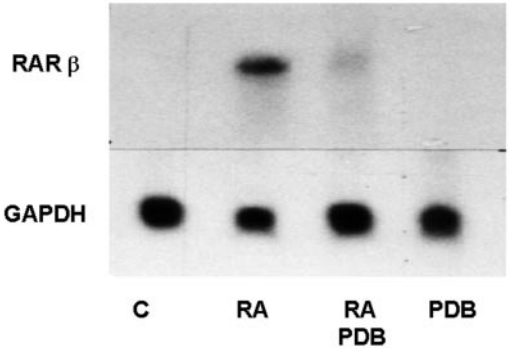

FIG. 8. **Down-regulation of PKC inhibits retinoic acid induction of RARβ mRNA.** B16 cells were treated with 10 μM phorbol dibutyrate for 24 h alone, 10 μM retinoic acid for 24 h alone, or with phorbol dibutyrate for 24 h prior to the addition of retinoic acid for 24 h. Cells were harvested, RNA was extracted, and the amount of RARβ was determined by Northern blotting as described in the text.

arrest and differentiation (36). Because PKC has been reported to phosphorylate RAR (29), we decided to investigate the potential interactions between PKC and RAR in our B16 melanoma model system.

Using transient transfection into B16 mouse melanoma cells of a construct containing the gene for 6X-His-RARα, we found that the encoded protein has multiple phosphorylated species in agreement with previous published results (37). However, in contrast to our expectations, activation or down-regulation of the endogenous PKC, through the use of short and long term phorbol dibutyrate treatment, did not change the phosphorylation status of the ectopically expressed RARα. This conclusion is based on the observation that manipulation of PKC activity/expression did not change the ratio of any of the phosphorylated species of RARα nor the amount of $^{32}P_i$ incorporated into the RARα when the data is corrected for the amount of RARα protein present in the sample. Our finding differs from that of Delmotte *et al.* (29), who showed that PKCα and γ were able to phosphorylate RARα *in vitro* on a single serine residue (Ser-157). Furthermore, they have shown that this phosphorylation site is at the dimerization interface and thus inhibits formation of RAR·RXR heterodimer and transcriptional activity. We have also found that PKCα was able to phosphorylate RARα *in vitro* (data not shown), however our present results suggest that this phosphorylation most likely does not occur *in vivo* in B16 melanoma cells.

Although activation of PKC did not alter the phosphorylation state of RARα, it did increase the amount of this protein. The increase was not due to increased promoter activity of the transfected plasmid, because the same promoter-driving β-galactosidase expression did not increase the expression of this transfected gene when the cells were treated for 2 h with phorbol dibutyrate. Although we did not measure RARα mRNA levels with or without addition of phorbol dibutyrate, it is unlikely that changes in RNA level can account for the increase in the amount of RARα protein, because it was detected within 30 min of addition of phorbol dibutyrate to the cells. Indeed, further investigation demonstrated that the increase could be

correlated exactly with an increase in the half-life of the RARα protein. It is clear that PKC enzyme activity is required for short term phorbol dibutyrate treatment to increase RARα protein. Both bisindolylmaleimide, a fairly specific inhibitor of PKC, and dominant-negative mutants of PKCα that lack enzyme activity, blocked the increase in RARα protein. Although PKCα is the major isozyme expressed in B16 cells (31), we are hesitant to claim that this PKC isotope is responsible for the increase in RARα, because overexpression of dominant-negative PKCα could block maturation of other isozyme by sequestering phosphoinositide-dependent kinase and thus blocking phosphorylation of the activation loop of the PKC enzyme (38).

Examination of the half-life of RARα in control and treated cells revealed that activation of PKC increased the half-life from 4 to 8 h, whereas down-regulation of PKC decreased the half-life to 3 h. This increase correlates quite well with the increased steady-state level of RARα in phorbol dibutyrate-treated cells. Several recent studies have shown that the RARs are degraded through the ubiquitin-proteasome pathway and that binding of ligand by the receptor accelerates its degradation (39–41). Because RARα did not appear to be directly phosphorylated by PKC *in vivo*, we speculate that one of the components of the ubiquitination-ligase system may be negatively regulated by PKC phosphorylation. Alternatively, PKC-mediated phosphorylation of associated co-repressors may block ligand-induced conformation change in the RARα, leading to stabilization of the protein. However, we consider this mechanism unlikely in light of the increased AF-2 activity in cells having activated PKC (see below).

In two studies PKC has been implicated in stabilizing protein half-life. Lee *et al.* (42) found that phosphorylation of EGF receptors by PKC decreased their degradation by promoting recycling back to the cell surface. Droms and Malkinson (43) reported that PKC βII inhibited the proteasome-dependent proteolysis of FUS nuclear proto-oncogene.

The increased amount of RAR protein in cells having activated PKC probably accounts for the increase in AF-2 activity that we observed. This was found with ectopically expressed RARα, β, and γ as well as with the endogenous RARs. However, the large decrease in AF-2 activity found in PKC down-regulated cells cannot be explained by the change in the receptor half-life from 4 to 3 h. We observed that the loss of PKC resulted in a large decrease in receptor DNA binding. This loss of receptor-DNA binding likely explains the drop in AF-2 activity. One possibility is that down-regulation of PKC affects heterodimer formation of RAR with RXR. It has been reported that RXR·RAR heterodimer formation is required for stable DNA binding (14). RXR is also a phosphoprotein (44). However, at present, it is not known whether it is phosphorylated by PKC *in vivo*. Down-regulation of PKC also inhibited induction of RARβ mRNA by retinoic acid. This is consistent with a loss of AF-2 activity and DNA binding of RAR in PKC-down-regulated cells.

In summary, in B16 mouse melanoma cells, activation of PKC increases the half-life of RARα, which correlates with enhanced AF-2 activity of all of the RAR. Down-regulation of PKC leads to markedly decreased AF-2 activity of the RAR and inhibition of retinoic acid induction of one of its target genes, *i.e.* RARβ. Phorbol dibutyrate promotes the formation of skin tumors when chronically applied to mouse skin (45, 46). We have shown that chronic treatment of B16 mouse melanoma cells with phorbol dibutyrate inhibits the ability of retinoic acid to induce growth arrest and differentiation in these cells (35, 47). Therefore, we hypothesize that phorbol dibutyrate may accomplish its tumor promoting activity, at least in part, by down-regulating PKC, which in turn blocks the ability of the RAR to transmit the signal of retinoids to regulate growth and/or differentiation.

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REFERENCES

1. Hofmann, C., and Eichele, G. (1994) in *The Retinoids, Biology, Chemistry and Medicine* (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) 2nd Ed., pp. 387–441, Raven Press, New York
2. Wilson, J. G., Roth, C. B., and Warkany, J. (1953) *Am. J. Anat.* **92,** 189–217
3. DeLuca, L. M. (1991) *FASEB J.* **5,** 2924–2933
4. Moreno-Manzano, V., Ishikawa, Y., Lucio-Cazana, J., and Kitamura, M. (1999) *J. Biol. Chem.* **274,** 20251–20258
5. Lotan, R. (1980) *Biochim. Biophys. Acta.* **605,** 33–38
6. Lupulescu, A. (1993) *Int. J. Vitam. Nutr. Res.* **63,** 397–405
7. Breitman, T. R., Selonick, S. E., and Collins, S. J. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 2936–2941
8. Mattei, M.-G., Riviere, M., Krust, A., Ingvarsson, S., Vennstrom, B., Islam, M. O., Levan, G., Kastner, P., Zelent, A., Chambon, P., Szpirer, J., and Szpirer, C. (1991) *Genomics* **10,** 1061–1066
9. Ishikawa, A., Umesono, K., Mangelsdorf, D. J., Aburtatani, H., Stanger, B. Z., Shibaski, Y., Imawari, M., Evans, R. M., and Takaku, F. (1990) *Mol. Endocrinol.* **4,** 837–843
10. Hoopes, C. W., Taketo, M., Ozato, K., Liu, Q., Howard, T. A., Linney, E., and Seldin, M. F. (1992) *Genomics* **14,** 611–616
11. Crettaz, M., Baron, A., Siegenthaler, G., and Hunziker, W. (1990) *Biochem. J.* **272,** 91–99
12. Heyman, R. A., Mangelsdorf, D. J., Dyck, J. A., Stein, R. B., Eichele, G., Evans, R. M., and Thaller, C. (1992) *Cell* **68,** 397–405
13. Levin, A. A., Struzenbecker, L. J., Kazmer, S., Bosakowski, T., Huelton, C., Allenby, G., Speck, J., Kratzeisen, C., Rosenberger, M., and Lovey, A. (1992) *Nature* **355,** 359–362
14. Kliewer, S. A., Umensono, J., Mangelsdorf, D. J., and Evans, R. M. (1992) *Nature* **335,** 446–449
15. Keller, H., Dreyer, C., Medin, J., Mahfoudi, A., Ozato, K., and Wahli, W. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2160–2166
16. Leid, M., Kastner, P., Lyons, R., Nakshari, H., Saunders, M., Zacharewski, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S., and Chambon, P. (1992) *Cell* **68,** 377–395
17. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) *Cell* **65,** 1255–1266
18. Xiao, Y., Desai, D., Quick, T. C., and Niles, R. M. (1996) *J. Cell. Physiol.* **167,** 413–421
19. Rochette-Egly, C., Oulad-Abdelghani, M., Staub, A., Pfister, V., Scheuer, I., Chambon, P., and Gaub, M.-P. (1995) *Mol. Endocrinol.* **9,** 860–871
20. Rochette-Egly, C., Adam, S., Rossignol, M., Egly, J. M., and Chambon, P. (1997) *Cell* **90,** 97–107
21. Rochette-Egly, C., Plassat, J.-L., Taneja, R., and Chambon, P. (2000) *Mol. Endocrinol.* **14,** 1398–1410
22. Taneja, R., Rochette-Egly, C., Plassat, J. L., Penna, L., Gaub, M. P., and Chambon, P. (1997) *EMBO J.* **16,** 6452–6465
23. Nishizuka, Y. (1986) *Science* **233,** 305–312
24. Ohno, S., Akita, Y., Hata, A., Osada, S. I., Kubo, K., Konno, Y., Akimoto, K., Mizuno, K., Saido, T., Kuroki, T., and Suzuki, K. (1991) *Adv. Enzyme Regul.* **31,** 287–303
25. Mellor, H., and Parker, P. J. (1998) *Biochem. J.* **332,** 281–292
26. Yuan, L. W., and Gambee, J. E. (2000) *J. Biol. Chem.* **275,** 40946–49851
27. Boulikas, T. (1995) *Crit. Rev. Eukaryot. Gene Expr.* **5,** 1–77
28. Tahayato, A., Lefebvre, P., Formstecher, P., and Dautrevaux, M. (1993) *Mol. Endocrinol.* **7,** 1642–1653
29. Delmotte, M.-H., Tahayato, A., Formstecher, P., and Lefebvre, P. (1999) *J. Biol. Chem.* **274,** 38225–38231
30. Radominska-Pandya, A., Chen, G., Czernik, P. J., Little, J. M., Samckyszyn, V. M., Carter, C. A., and Nowak, G. (2000) *J. Biol. Chem.* **275,** 22324–22330
31. Rosenbaum, S., and Niles, R. M. (1992) *Arch. Biochem. Biophys.* **294,** 123–129
32. Desai, S. H., Boskovic, G., Eastham, L., Dawson, M., and Niles, R. M. (2000) *Biochem. Pharmacol.* **59,** 1265–1275
33. Sucov, H. M., Murakami, K. K., and Evans, R. M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **68,** 5392–5396
34. Gruber, J. R., Desai, S., Blusztajn, J. K., and Niles, R. M. (1995) *Exp. Cell Res.* **221,** 377–384
35. Niles, R. M., Mahalingam, H., Vaughn, J., Novotny, J., and Gruber, J. R. (1996) *J. Cell. Physiol.* **168,** 549–558
36. Gruber, J., Ohno, S., and Niles, R. M. (1992) *J. Biol. Chem.* **267,** 13356–13360
37. Gaub, M. P., Rochette-Egly, C., Lutz, Y., Ali, S., Matthes, H., Scheuer, I., and Chambon, P. (1992) *Exp. Cell Res.* **201,** 335–346
38. Parekh, D. B., Ziegler, W., and Parker, P. J. (2000) *EMBO J.* **19,** 496–503
39. Zhu, J., Gianni, M., Kopf, E., Chelbl-Alix, M., Koken, M., Quignon, F., Rochette-Egly, C., and de The, H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14807–14812
40. Kopf, E., Plassat, J.-L., Vivat, V., de The, H., Chambon, P., and Rochette-Egly, C. (2000) *J. Biol. Chem.* **275,** 33280–33288
41. Boudjelal, M., Wang, Z., Voorhees, J. J., and Fisher, G. J. (2000) *Cancer Res.* **60,** 2247–2252
42. Bao, J., Alroy, I., Waterman, H., Schejter, E. D., Brodie, C., Gruenberg, J., and Yarden, Y. (2000) *J. Biol. Chem.* **275**, 26178–26186
43. Perrotti, D., Iervolino, A., Cesi, V., Cirinna, M., Lombardini, S., Grassilli, E., Bonatti, S., Claudio, P. P., and Calabretta, B. (2000) *Mol. Cell. Biol.* **20**, 6159–6169
44. Lee, H.-Y., Suh, Y.-A., Robinson, M. J., Clifford, J. L., Hong, W. K., Woodgett, J. R., Cobb, M. H., Mangelsdorf, D. J., and Kurie, J. M. (2000) *J. Biol. Chem.* **275,** 32193–32199
45. Droms, K. A., and Malkinson, A. M. (1991) *Mol. Carcinogenesis* **4,** 1–2
46. Fournier, A., and Murray, A. W. (1987) *Nature* **330,** 767–769
47. Niles, R. M., and Loewy, B. P. (1989) *Cancer Res.* **49,** 4483–4487