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Stimulation of Melanogenesis in a Human Melanoma Cell Line by Bistratene A

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ABSTRACT. The polyether toxin, bistratene A, induced morphological and functional differentiation of a human melanoma cell line (MM96E). The cells became blocked at the G2/M transition and elaborated a number of processes. Tyrosinase activity and melanin content were substantially increased. Northern blot analysis showed up-regulation of mRNA for several genes known to be involved in melanin biosynthesis (pmel17, pmel34, and tyrosinase related proteins, TRP-1 and TRP-2). Bistratene A induced the phosphorylation of several proteins as assessed by 2D gel electrophoresis and one of these was identified as stathmin (oncoprotein 18), a cell-cycle regulated phosphoprotein. Bistratene A specifically induced the translocation of protein kinase C δ (PKCδ) from a soluble to a particulate fraction without affecting other isoforms. These results implicate a role for protein kinase C δ in the induction of differentiation of this human melanoma cell line. BIOCHEM PHARMACOL **55**;10:1691–1699, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. melanogenesis; bistratene A; protein kinase C delta; stathmin; cell cycle

Malignant melanoma remains one of the most difficult cancers to treat successfully. Our understanding and future prospects of treating this disease would be greatly enhanced by a detailed knowledge of mechanisms governing differentiation of melanoma cells. Melanin biosynthesis is a unique and widely studied aspect of melanoma differentiation, in particular the regulation of the rate-limiting enzyme, tyrosinase. Tyrosinase is encoded by a gene located at the *albino* locus [1]. Other TRPs§ (TRP-1 and TRP-2 [DOPAchrome tautomerase]) are coded by the *brown* locus [2] and the *slaty* locus [3], respectively. The pmel17 protein is coded by a gene which maps to the *silver* locus and it has been suggested that it is a melanosomal matrix protein [4]. Kwon has proposed that genes regulating melanin biosynthesis be classified into the tyrosinase gene family and the pmel 17 gene family, with the former, comprising tyrosine and dopachrome tautomerase catalysing proximal steps of the Mason-Raper pathway and the latter catalysing distal steps of the biosynthetic pathway [5]. Pmel 17 gene expression is more closely correlated with the level of melanin than is tyrosinase expression [6]. It has been suggested that the key signal transducing enzyme PKC has a role in melanogenesis because diacylglycerol induces human melanogenesis in cultured human melanocytes [7]. The β isoform of PKC has been shown to regulate melanogenesis by activating tyrosinase [8], and pigmentation of S91 Cloudman mouse melanoma induced by α-melanocyte stimulating hormone correlated with up-regulation of the β-isoform of PKC [9]. In contrast, PKCα has been implicated in the regulation of melanogenesis in B16 mouse melanoma cells [10]. PKC has been shown to play an important role in the transformation of melanocytes to melanomas, and PKCβ is missing from many melanoma cells but present in melanocytes [11,12]. The proliferation of melanocytic cells has been associated with down-regulation of PKC α, δ, and ϵ isoforms [13]. PKC is also implicated in the development of metastases since a PKC inhibitor, calphostin C, reduces lung colonisation of a B16 melanoma [14]. In this paper, we show that bistratene A, a specific activator of PKCδ [15], inhibits growth and induces the transcription of several genes involved in melanogenesis in a human melanoma cell line.

MATERIALS AND METHODS

Materials

Bistratene A was isolated and purified by HPLC from the ascidian *Lissoclinum bistratum* as previously described [16]. TPA was purchased from Sigma. [^{32}P] orthophosphate was obtained from Dupont/NEN. Antibodies to PKC isoforms were purchased from Transduction Laboratories or Santa Cruz Laboratories. Ampholytes, pH range 3–10 and 5–7, were from Pharmacia. Ultrapure electrophoresis materials were obtained from Bio-Rad, and all other reagents were

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§ *Abbreviations:* GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NFF, neonatal foreskin fibroblasts; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; TRP, tyrosinase related protein.

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analytical grade. Horseradish peroxidase-labelled, and FITC-labelled anti-rabbit Ig, produced in donkey, were obtained from Amersham. Chemiluminescence reagents were from DuPont (Renaissance Kit).

Cell Culture and Labelling

MM96E and MM418c1 melanoma cell lines have been described [17, 18] and were routinely cultured in 5% CO_2/air at 37° in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal calf serum. NFF were obtained from Prof. Kay Ellem of this Institute. Neonatal melanocytes were cultured in the presence of 100 ng/mL of TPA and 2 ng/mL of cholera toxin. Monthly assays for mycoplasma by Hoechst 33258 stain [19] were negative. For labelling experiments, one small flask (25 cm^2) of confluent cells (3×10^6 cells) was washed twice with phosphate-free MEM and incubated for 30 min at 37° in the same medium. Carrier-free ^{32}P (0.1 mCi in 1 mL) was added and the cells equilibrated for 3 hr at 37°. Bistratene A (100 nM) was added and incubation continued for 1 hr. The medium was removed and the cells washed twice with stopping buffer (20 mM of potassium phosphate, pH 7.4, 50 mM of sodium fluoride, 5 mM of sodium pyrophosphate, 100 μM of sodium vanadate, 5 mM of EDTA (ethylene diamine tetraacetic acid), 50 ng/mL of aprotinin, 1 μM of pepstatin, 1 mM of phenylmethylsulfonyl fluoride. The cells were harvested by incubation in 1 mL of trypsin 0.25% (w/v) in PBS, transferred to an Eppendorf tube and centrifuged for 30 sec in a microfuge. They were washed with 0.5 mL of stopping buffer and resuspended in 50 μL of isoelectric focussing sample buffer [9.2 M of urea, 2% nonidet NP-40 (v/v), 1% dithiothreitol (w/v), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-propane-sulfonate), Ampholines pH range 3–10 1:16 dilution (6.25%)]. Samples were stored at −70°.

Cell Survival Assay

The survival of MM96E cells after treatment with bistratene A was assessed using incorporation of radiolabelled thymidine. This method gives similar results to clonogenic assays with a range of agents. Cells plated 20 hr previously in 100 μL of medium (30,000 cells/mL) in 96-well microtitre plates were treated with drug and incubated for 6–7 days. The medium was replaced with 100 μL of fresh medium containing 2 μCi/mL of [^3H]thymidine, incubated for 4 hr, and then the cells were detached with trypsin before harvest onto glass fibre mats with water. Incorporation of label (5,000–20,000 cpm in controls) was determined with a scintillation counter and expressed as a percentage of untreated controls. Background was 10–15 cpm and the standard deviation was less than 10% ($N = 3$).

Cell-Cycle Analysis

Cells (~10^6) were harvested at various times after bistratene A treatment by centrifugation and treated with 0.25 mL of propidium iodide at 50 μg/mL containing 1 mg/mL of ribonuclease and 0.2% Triton X-100. After incubation on ice for 30 min, samples were analysed on a Becton Dickinson FACScan instrument. The percentage of cells in each cell-cycle phase was estimated using Lysis II software (Becton Dickinson).

Preparation of Cell Extracts and Subcellular Fractionation

One medium flask (75 cm^2) of MM96E cells (~10^7 cells) was treated with 100 nM of bistratene A for 1 hr at 37°. The cells were trypsinised to remove them from the plastic surface and centrifuged at 1,000 *g* for 5 min. The cell pellet was washed twice with PBS by centrifugation. The final pellet was resuspended in 0.2 mL of ice-cold homogenisation buffer A (20 mM of Tris, pH 8.0, 10 mM of EGTA, 2 mM of EDTA, 10 mM of β-mercaptoethanol, and "Complete" protease inhibitor cocktail from Boehringer Mannheim) as described by Oka *et al.* [20], except that homogenisation was carried out using 30 strokes in a Dounce homogeniser instead of sonication. The homogenate was centrifuged at 100,000 *g* for 60 min. The resulting pellet was lysed in 0.2 mL of buffer B (buffer A containing 0.5% Triton X-100) by sonication for 3 × 10 s (50% duty cycle) at 4°, and the supernatant from a 30-min microfuge spin retained as the particulate fraction. Protein concentrations were determined by the Bradford assay and equal amounts loaded onto SDS-PAGE.

SDS-PAGE

One dimensional SDS-PAGE gels (10% acrylamide) were run in a Bio-Rad Mini Protean II apparatus. Prestained markers were added to one lane and the gels blotted onto nitrocellulose membrane (Hybond C, Amersham) using a Trans-Blot module (Bio-Rad) and CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer (10 mM of CAPS, pH 11, and 10% methanol).

Two dimensional gel electrophoresis was carried out using the Pharmacia LKB Multiphor system. The first dimension (isoelectric focussing) was run on Immobiline dry strips (pH 3.0–10.5) and precast gradient SDS-PAGE gels (ExcelGel SDS gradient 8–18) were used for the second dimension. Molecular weights and isoelectric points were established with reference to Bio-Rad 2D markers.

Determination of Tyrosinase (Dopa Oxidase) Activity and Melanin Content

Cell pellets were treated with lysis buffer (200 μL of 1% Triton X-100 in 50 μM of phosphate, pH 6.8), sonicated and microfuged to remove melanin and debris. Samples of supernatant (20 μL) were incubated at room temperature in microtitre wells with 100 μL of 50 mM of phosphate buffer (pH 6.8) containing 7.8 mM of L-dopa. The absorbance change was monitored at 495 nm on an ELISA

reader at 5-min intervals. Activity was calculated as A_{495}/min/mg of protein. The level of melanin was determined by A_{400} of cell pellets solubilised in 1 N of NaOH.

Preparation of Messenger RNA

MM96E cells were grown in several 150 cm^2 flasks and approx 2×10^8 cells used for isolation of mRNA. Cells were treated with bistratene A (100 nM) for 1, 3, and 5 days. After the appropriate incubation time, the cell monolayers were trypsinised and pelleted by centrifugation. The pellet was washed twice in STE buffer (50 mM of Tris-HCl, pH 7.6, 2 mM of EDTA, 100 mM of NaCl) and the cells then lysed in 20 mL of the same buffer containing 0.5% SDS and the mRNA isolated by standard procedures [21]. The mRNA was loaded onto denaturing formaldehyde containing agarose gels, transferred immediately to nitrocellulose (Hybond N, Amersham) by capillary transfer and cross-linked by UV irradiation.

Probes were labelled by random priming using [^{32}P]dCTP. Probes for melanin synthesis genes were as follows: human TRP-1, pHTα2 [22]; human tyrosinase, pmel34 [23]; mouse TRP-2, pTRP2A [3]; human pmel17: pBSmel17 was generated by cloning a PCR fragment made from human cDNA with amplimers based on the human pmel17 sequence [24] and was a gift from Rick Sturm. To normalise for loading the blots were probed for β-actin. Analysis of the Northern blots was carried out on a Phosphorimager (Molecular Dynamics) using Image Quant software.

RESULTS

Bistratene A Induces Morphological Changes and Accumulation of Cells in G2/M

Cell survival curves for several different cell types in the presence of bistratene A are shown in Fig. 1. The IC_{50} for both MM418c1 and neonatal foreskin fibroblasts is 20 nM and for MM96E cells is 80 nM. On the other hand, HeLa cells are resistant to the actions of bistratene A with little effect on survival at 100 nM. The insensitivity of these cells is probably due to their drug resistant phenotype. When assayed using a colorimetric assay based on the binding of sulforhodamine to protein [25], the IC_{50} values for B16 (20 nM, data not shown) and NFF cells were similar to that determined by incorporation of tritiated thymidine; however, the IC_{50} for MM96E cells was much higher—about 4 μM (data not shown)—suggesting that these cells are not dying, but differentiating. A concentration of 50 to 100 nM was used throughout this work. After several days of treatment, the MM96E cells became more rounded and refractile and elaborated numerous processes reminiscent of neurones. Some multinucleated cells are also apparent (Fig. 2). The cells also became darker in colour, suggesting increased melanin synthesis. These changes suggested that the MM96E cells undergo differentiation in the presence of bistratene A. Human melanocytes require high cell density for growth and their survival could not be determined by these methods. However, growth of melanocytes as assessed by cell number was 53% of controls after treatment with bistratene A for 3 days.

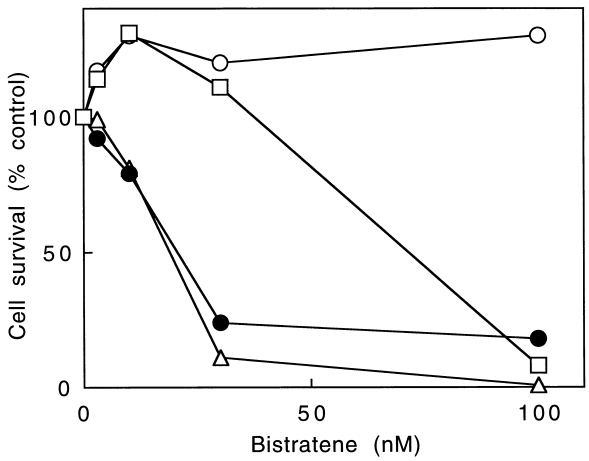

FIG. 1. Incorporation of [^{3}H]thymidine in the presence of bistratene A. Cells were plated into 96-well plates as described in Materials and Methods. Six to seven days after treatment with the toxin, the medium was replaced with fresh medium containing [^{3}H]thymidine, incubated for 4 hr, then assayed for incorporation of label into DNA. The standard deviation was less than 10% (N = 3). (○) HeLa; (□) MM96E; (●) MM418; (Δ) NFF.

Cell-cycle analysis of MM96E cells showed that there was a considerable increase in the G2 population 3 days after treatment with bistratene A at 100 nM (Fig. 3). On the other hand, HeLa cells do not show this accumulation in G2 (data not shown). In a previous study, we showed that HL-60 cells accumulated in G2 and a polyploid peak was evident which indicated that many cells were undergoing a further round of DNA replication without mitosis [26]. Only a very small polyploid peak is present in the MM96E cells; however, multinucleated cells are visible in stained preparations of the cells (Fig. 2b). In contrast to the effects of bistratene A, TPA did not cause any change in the cell-cycle distribution of these cells (data not shown). The lack of response of HeLa cells to bistratene A is most likely related to their drug resistant status.

Melanin Synthesis and Tyrosinase Activity are Induced by Bistratene A

Because the cells became noticeably darker after 3 days of treatment with bistratene A, we determined the effect of bistratene A on melanin synthesis and tyrosinase activity. The results are shown in Table 1. The amount of melanin increased approximately two-fold on treatment with bistratene A for 3 days. The tyrosinase activity also increased two-fold at this time. No increase in tyrosinase activity was observed in the mouse B16 melanoma cell line and these cells also did not show altered morphology or increased melanin production after bistratene A treatment. Tyrosinase activity was increased in melanocytes at 3 days after bistratene A treatment; however, this was not accom-

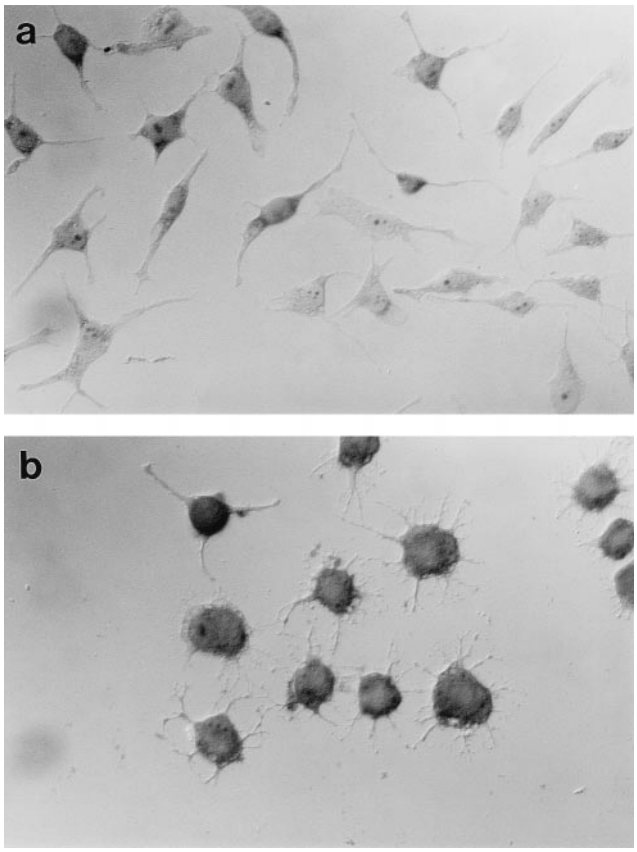

FIG. 2. Morphology of MM96E cells. Cells were grown on coverslips, fixed with 4% formalin in PBS for 20 min, and then photographed using a Zeiss Axioskop DIC/phase contrast microscope at 400× magnification; (a) untreated; (b) treated with bistratene A (100 nM) for 3 days.

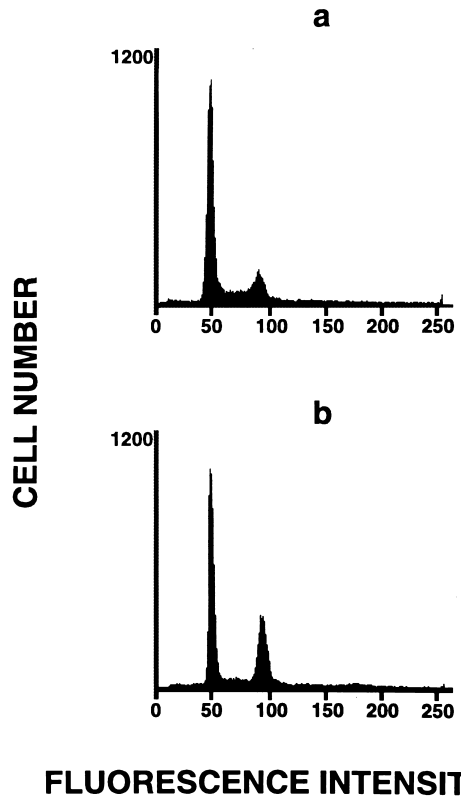

FIG. 3. Cell-cycle analysis of MM96E cells. Cells (approx. 10^6) were harvested by centrifugation and treated with propidium iodide as described in Materials and Methods. They were analysed on a Becton Dickinson FACScan instrument using Lysis II software; (a) untreated; (b) treated with bistratene A (100 nM) for 3 days. The percentage of cells in G2 was 12% for untreated and 32% for bistratene A treated.

panied by an increase in melanin synthesis. In MM96E cells, TPA treatment also resulted in a two-fold increase in tyrosinase activity, but no change in melanin content was observed (data not shown).

Messenger RNA levels for enzymes involved in melanin synthesis were examined by Northern blotting and the results are presented quantitatively in Table 2. The levels of mRNA for pmel34 (tyrosinase), pmel17, TRP-1, and TRP-2 were increased several fold relative to β-actin by bistratene A treatment in MM96E cells. GAPDH message was also shown to increase. Interestingly, in B16 and 418c1 melanoma cells which do not show an increase in melanin synthesis after bistratene A treatment, there was no increase in the levels of mRNA for TRP-1 or TRP-2.

Bistratene A Induces the Phosphorylation of Several Proteins and Redistribution of PKCδ

Bistratene A has been shown to increase specific protein phosphorylation in HL60 cells by 2D gel electrophoresis [27]. We carried out similar experiments in MM96E cells with a view to the eventual determination of phosphoproteins which may play a role in the differentiation process. The results are shown in Fig. 4. For comparison, 2D gels of TPA-treated MM96E cells are also shown. The phosphorylation of several proteins is increased in MM96E cells in the presence of bistratene A (labelled 1–7 in Fig. 4b). One of these proteins is most likely to be stathmin, judging from its molecular weight and isoelectric point and the pattern in the gel (spots #12, 13). This was confirmed using Western blotting (Fig. 5) with a polyclonal antibody to stathmin. The forms of stathmin which are increased after bistratene A treatment represent α2, α3, and β1 according to the scheme proposed by Beretta *et al.* [28]. TPA also induced the phosphorylation of several proteins (spots 3,4,7–13) including stathmin. A slower migrating form of stathmin (open arrow, Fig. 4c) was induced in the presence of TPA, but not of bistratene A, in these cells. The slower migrating form present in TPA-treated samples could correspond to the α_2^1 or α_1^2 forms described by [28]. The identity of the other numbered proteins is currently unknown.

We have recently shown that bistratene A leads to activation of a specific isoform of PKC namely PKCδ in HL60 cells [15]. PKC activation is often measured as a translocation from one cellular compartment to another. We have previously shown the translocation of PKCδ after bistratene A treatment in HL-60 cells [15] and NFF

TABLE 1. Induction of tyrosinase activity and melanin in melanoma cells and melonocytes by bistratene A

Time after treatment (100 nM bistratene A)	% Control				
	Tyrosinase			Melanin	
	MM96E*	B16*	Melanocytes*	MM96E†	Melanocytes†
1 Day	121‡	ND§	ND	105	ND
2 Days	200	71	169	187	104
3 Days	138	ND	ND	225	ND

*Control levels: 0.0053 (MM96E); 0.0041 (B16); 0.0065 (melanocytes) A_{495}/min/mg of protein.
†Control levels: 0.71 (MM96E) and 5.5 (melanocytes) μg/mg of protein.
‡Means of triplicates.
§Not determined.

fibroblasts [29]. Therefore, we examined the subcellular distribution of PKC isoforms in MM96E cells by Western blotting using PKC-isoform specific antibodies (Fig. 6a). There was no change in the distribution of PKC α,β,ϵ,ι,ζ, whereas there was a definite movement of PKCδ to the particulate fraction at 10 min. The amount of enzyme in the particulate fraction was determined by densitometry in three separate experiments and increased ~three-fold (data not shown). Translocation of PKCδ was also seen in other cell types; e.g. the B16 melanoma cells (Fig. 6b), which did not respond to bistratene A by increasing melanin synthesis.

DISCUSSION

The results presented in this study show that bistratene A induces morphological and functional differentiation of the lightly pigmented human melanoma cell line MM96E. The cells became blocked at the G2/M transition and elaborated a number of processes. Tyrosinase activity and melanin content were substantially increased. Northern blot analysis showed the up-regulation of mRNA for several genes involved in melanin biosynthesis (pmel17, pmel34, TRP-1 and TRP-2). Previous studies showed that the differentiating agents butyrate and DMSO, increased tyrosinase activity in these cells but down-regulated expression of TRP-1 [30]; the latter effect was also found in melanoma cells with the differentiating agent hexamethylene bisacetamide [31]. Therefore, Bistratene A appears to be unique in up-regulating all pigmentation markers tested, including melanin synthesis, in lightly pigmented melanoma cells.

Studies in melanocytes showed that melanogenesis was

TABLE 2. Northern blot analysis of the expression of genes involved in melanin biosynthesis

Probe	Cell line	Untreated	1 day	3 day	5 day
TRP-1	MM96E	1	2.5	5.9	3.4
	MM418c1	1	1.3		
	B16	1	1.5	1.2	
TRP-2	MM96E	1	3.2	12.9	3.6
	MM418c1	1	1.1	0.6	0.6
pmel17	MM96E	1	3.3	7.4	4.8
pmel34	MM96E	1	2	3.4	7.6
GAPDH	MM96E	1	2.6	2.2	4.7
	MM418c1	1	1.6	1	2.6
	B16	1	2.8	4.8	3.7

Results expressed as fold increase over untreated controls, average of three experiments normalised to β-actin.

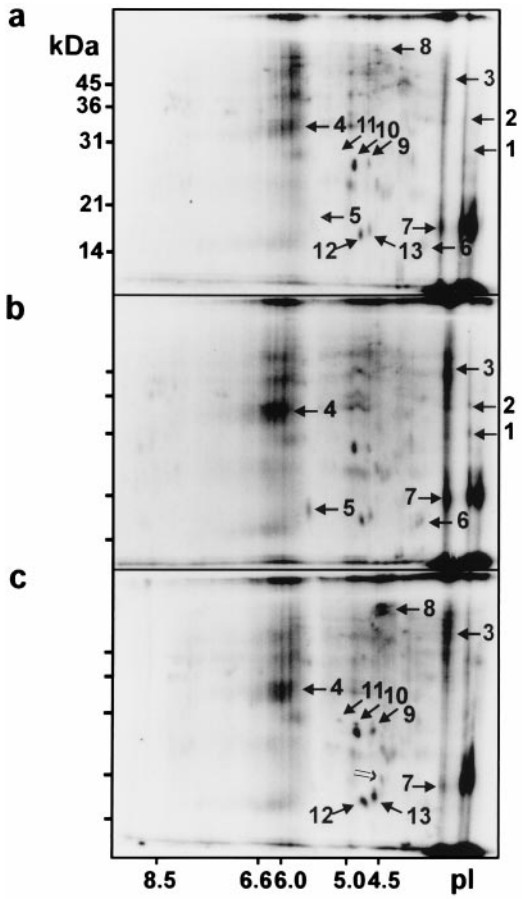

FIG. 4. 2D SDS-PAGE of MM96E phosphoproteins. MM96E cells were labelled with ^{32}P for 3 hr and then treated with bistratene A (100 nM) or TPA (100 nM) for 1 hr. After trypsinisation and washing, the cell pellet was resuspended in IEF sample buffer and analysed by 2D-SDS-PAGE. The gels were fixed, dried, and subjected to Phosphorimager analysis; spots which are increased by either treatment are numbered 1–13; (a) untreated; (b) bistratene A-treated; (c)TPA treated.

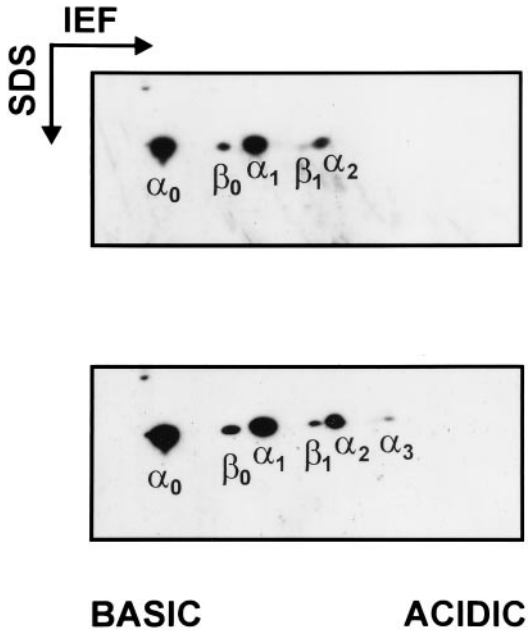

FIG. 5. 2D Western analysis of stathmin forms. MM96E cells were treated with bistratene A and extracts separated by 2D SDS-PAGE. The gels were blotted onto nitrocellulose membrane and the various forms of stathmin detected with a polyclonal antistathmin antibody. The different forms of stathmin are labelled according to the scheme of Beretta *et al.* [28].

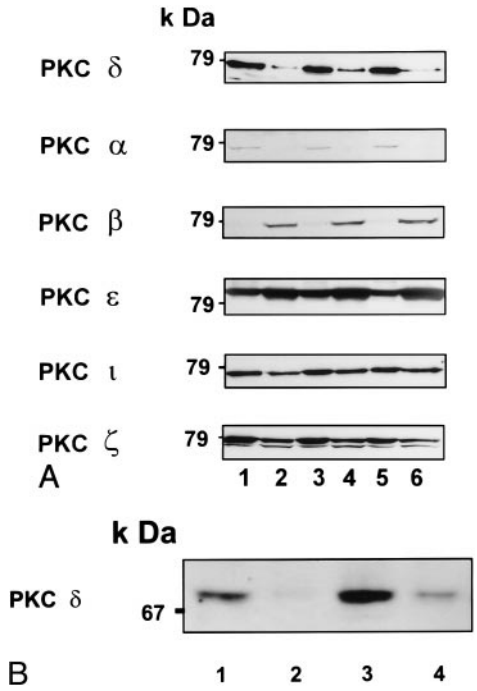

FIG. 6. Effect of bistratene A on subcellular distribution of PKC isoforms. (a) MM96E cells were treated with 100 nM bistratene A for 10 min or 1 hr and then separated into soluble and particulate fractions as described under Materials and Methods. The fractions were separated by SDS-PAGE and blotted onto nitrocellulose membrane for Western analysis with PKC isoform-specific antibodies. After blotting, the membranes were briefly stained with Ponceau S to ensure equal loading. The position of prestained SDS-PAGE markers (Bio-Rad) is indicated. This experiment was repeated three times with similar results. (1) untreated, soluble; (2) untreated, particulate; (3) bistratene A 10 min, soluble; (4) bistratene A 10 min, particulate; (5) bistratene A 1 hr, soluble; (6) bistratene A 1 hr, particulate. (b) B16 mouse melanoma cells were analysed as in (a) above, (1) untreated, soluble; (2) untreated, particulate; (3) bistratene A 10 min, soluble; (4) bistratene A 10 min, particulate.

increased after UV irradiation presumably as a result of increased tyrosinase activity concomitant with a block in the G2/M phase [32]. In that study, there was no effect on the amount of tyrosinase or TRP-1. The effect of bistratene A also contrasts with that of the phorbol ester, TPA, in MM96E cells, in that TPA did not affect cell-cycle distribution, and tyrosinase activity was increased without affecting melanin levels (data not shown). The increase in tyrosinase activity induced by bistratene A in this study initially paralled the increase in mRNA expression; however, this was not the case at 3 days after treatment. The relationship between tyrosinase activity, expression, and melanogenesis is likely to be complex, involving post-translational regulation of enzyme activity [33–35]. Interestingly, mRNA levels for GAPDH also increased on treatment with bistratene A. An increase in GAPDH expression has also been observed on stimulation of T-helper lymphocytes with interleukin-2 (IL-2) [36] and on treatment of human mammary epithelial cells with 1,25-dihydroxyvitamin D3 [37]. The significance of the increase in GAPDH expression is unclear.

The phorbol ester TPA stimulates the growth of cultured normal melanocytes [38], yet inhibits the growth of many melanoma derived cell lines [11, 39–43]. TPA has long been recognised as an activator of PKC and the apparently paradoxical effects of TPA in a number of systems can be explained by the fact that PKC comprises a family of at least twelve isoenzymes [44]. Information on the role of individual PKC isoforms in cell cycle control, differentiation and growth inhibition is limited. Growth inhibition of the SK-Mel-173 melanoma cell line by TPA has been shown to be associated with down-regulation of PKCβ [43]. This down-regulation did not occur in a TPA-resistant variant line. The work of Yamanishi *et al.* suggests that loss of PKCβII occurs in melanocyte transformation [12, 45]; however, this is not always the case. In the case of MM96E cells, TPA did not induce changes in morphology or melanin synthesis, and there was no change in the cell-cycle distribution at 3 days after treatment (data not shown). Because we have shown that bistratene A leads to the activation of specifically the delta isoform of PKC in HL-60 cells [15] and in human fibroblasts [29], we examined the activation of PKC isoforms in MM96E cells by using translocation from soluble to particulate fractions. Only the delta isoform was affected by bistratene A treatment consistent with our results in HL-60 cells and fibroblasts [15, 29]. In cells which did not respond to bistratene A by increasing melanogenesis in this study, e.g. the B16 mouse melanoma cells, there was still translocation of

PKCδ from a soluble to a particulate fraction. Thus, the consequences of activation of the enzyme, e.g. triggering of the differentiation programme, must depend on other additional factors. B16 melanoma cells already synthesise large amounts of melanin and, hence, different mechanisms of regulation are likely to exist in these cells. The regulation of melanogenesis by PKC in B16 cells has been reported [10] to be associated with the PKCα isoform. The ability of phorbol esters to down-regulate PKCα correlated with their potency in inhibiting tyrosinase and TRP-1 expression. In mouse S91 Cloudman cells, melanogenesis induced by α-MSH is associated with an up-regulation of PKCβ [9], which has been previously shown to specifically activate tyrosinase [8]. Thus, it appears likely that the regulation of melanogenesis by PKC is species and cell-type specific.

Our results support a role for PKCδ in the differentiation of the MM96E melanoma cell line. The delta isoform has also been found in a number of other systems to have a role mainly in growth inhibition and differentiation. Overexpression of PKCδ in NIH3T3 fibroblasts and human glioma cells results in growth inhibition [46]. In unstimulated smooth muscle cells the δ isoform is associated mostly with the membrane fraction and is lost on transformation [47]. The differentiation of 32D myeloid cells has also been shown to be dependent on PKCδ [48].

Bistratene A induced the phosphorylation of several proteins as assessed by 2D gel electrophoresis. So far only one of these has been identified as stathmin. The phosphorylation of stathmin seen in the presence of bistratene A is interesting in light of the cell-cycle effects. Stathmin was identified initially because of its elevated expression in leukemias and its complex phosphorylation pattern in response to various activating signals [49]. Phosphorylation site-mapping studies showed that the protein is phosphorylated on Serines 16, 25, 38 and 63 and that phosphorylations of all four residues fluctuate during the cell cycle. The cyclin-dependent kinases (CDKs) were shown to be involved in the phosphorylation of Ser25 and Ser38, Ser25 is a major target of the mitogen-activted protein kinase family and Ser16 is a target for the Ca^{2+}/calmodulin-dependent kinase Gr [50,51]. Elegant site-directed mutagenesis studies demonstrated that overexpression of stathmin, with both CDK sites mutated to alanine, resulted in the accumulation of cells with a G2/M content of DNA and endoreduplication [50]. The same phenotype was observed after mutation of both Ser16 and Ser63 and it is thought that phosphorylation of Ser16 and Ser63 is essential during G2/M transition. The observed phenotype of the Ser25 and Ser38 double mutants is caused by interference with phosphorylation of Ser16 and Ser63 [50]. Recently, stathmin was identified as a microtubule catastrophe factor and it is thought to be involved in the physiological regulation of mitotic microtubule dynamics [52, 53] through phosphorylation of Ser16 [51]. Because the phenotype of bistratene A-treated cells is similar to that of the CDK site mutants, it will be of interest to determine the sites phosphorylated in the presence of bistratene A and whether PKCδ itself or a downstream kinase is responsible. Interestingly, stathmin is also phosphorylated in the presence of TPA in MM96E cells; however, TPA treatment does not lead to a block in G2/M. Further studies will be required to clarify the function of the different forms of stathmin and whether other mechanisms are involved in the G2 block seen in the presence of bistratene A. Stathmin has also been shown to be essential for nerve growth factor stimulated differentiation of PC12 cells [54]. The identification of other proteins phosphorylated in the presence of bistratene A will be important to understanding the differentiation process.

In conclusion, our studies point to a role for PKCδ in melanogenesis and differentiation of MM96E human melanoma cells; and bistratene A should be a useful tool to further investigate these processes.

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References

1. Kwon BS, Haq AK, Wakulchik M, Kestler D, Barton DE, Franke U, Lamoroux ML, Whitney, JB and Halaban R, Isolation, chromosomal mapping and expression of the mouse tyrosinase gene. *J. Invest. Dermatol.* **93:** 589–594, 1989.
2. Jackson IJ, A cDNA encoding tyrosinase-related protein maps to the brown locus in mice. *Proc. Natl. Acad. Sci. USA* **85:** 4392–4396, 1988.
3. Jackson IJ, Chambers DM, Tsukamoto K, Copeland NG, Gilbert DJ, Jenkins NA and Hearing V, A second tyrosinase-related protein, TRP-2 maps to and is mutated at the mouse slaty locus. *EMBO J.* **11:** 527–536, 1992.
4. Kobayashi T, Urabe K, Orlow SJ, Higashi K, Imokawa G, Kwon BS, Potter B and Hearing VJ, The Pmel 17/silver locus protein. Characterization and investigation of its melanogenic function. *J. Biol. Chem.* **269:** 29198–29205, 1994.
5. Kwon BS, Pigmentation genes: the tyrosinase gene family and the Pmel17 gene family. *J. Invest. Dermatol.* **100:** Suppl 134S–140S, 1993.
6. Kwon BS, Halaban R, Kim GS, Pomerantz S, Haq AK, A melanocyte specific cDNA clone whose expression is inducible by MSH and IBMX. *Mol. Biol. Med.* **4:** 339–356, 1987.
7. Gordon PR and Gilchrest BA, Human melanogenesis is stimulated by diacylglycerol. *J. Invest. Dermatol.* **93:** 700–702, 1989.
8. Park H-Y, Russakovsky V, Ohno S and Gilchrest BA, The β-isoform of protein kinase C stimulates human melanogenesis by activating tyrosinase in pigment cells. *J. Biol. Chem.* **268:** 11742–11749, 1993.
9. Park H-Y, Russakovsky V, Ao Y, Fernandez E and Gilchrest BA, Alpha-melanocyte stimulating hormone-induced pigmentation is blocked by depletion of protein kinase C. *Exp. Cell Res.* **227:** 70–79, 1996.
10. Mahalingam H, Vaughn J, Novotny J, Gruber JR and Niles RM, Regulation of melanogenesis in B16 mouse melanoma cells by protein kinase C. *J. Cell. Physiol.* **168:** 549–558, 1996.
11. Powell MB, Rosenberg RK, Graham MJ, Birch M, Yamanishi DT, Buckmeier JA and Meyskens Jr FL, Protein kinase C β expression in melanoma cells and melanocytes: differential expression correlates with biologic responses to 12-O-tetra-

decanoylphorbol 13 acetate. *J. Cancer Res. Clin. Oncol.* **119:** 199–206, 1993.
12. Yamanishi DT and Meyskens Jr., Alterations in gene expression and signal transductions in human melanocytes and melanoma cells. *Crit. Rev. Oncogen.* **5:** 429–450, 1994.
13. Brooks G, Goss MW, East JA and Hart IR, Growth of melanocytic cells is associated with down-regulation of protein kinase C α, δ and ϵ isoforms. *J. Biol. Chem.* **268:** 23868–23875, 1993.
14. Liu B, Renaud C, Nilson KK, Chen YQ, Bazaz R, Kowynia J, Timar J, Diglio CA and Honn KV, Protein kinase C inhibitor calphostin C reduces B16 amelanotic melanoma cell adhesion to endothelium and lung colonisation. *Int. J. Cancer* **52:** 147–152, 1992.
15. Griffiths G, Garrone B, Deacon E, Pongracz J, Owen P, Meade G, Watters D and Lord J, The polyether Bistratene, A activates protein kinase C-δ and induces growth arrest in HL-60 cells. *Biochem. Biophys. Res. Commun.* **222:** 802–808, 1996.
16. Degnan B, Hawkins CJ, Lavin MF, McCaffrey E, Parry D and Watters D, Novel cytotoxic compounds from the ascidian *Lissoclinum bistratum*. *J. Med. Chem.* **32:** 1355–1359, 1989.
17. Whitehead R and Little JH, Tissue culture studies on human malignant melanoma. *Pigment Cell* **1:** 382–289, 1973.
18. Maynard K and Parsons PG, Cross-sensitivity of methylating agents, hydroxyurea, and methotrexate in human tumour cells of the MER-phenotype. *Cancer Res.* **46:** 5009–5013, 1986.
19. Chen TR, *In situ* detection of mycoplasma contamination in cell culture by fluorescent Hoechst 33258 stain. *Exp. Cell Res.* **104:** 255–262, 1977.
20. Oka M, Ando H, Kikkawa U and Ichihashi M, Differential down-regulation of Protein kinase C subspecies in normal human melanocytes: possible involvement of the ζ subspecies in growth regulation. *J. Invest. Dermatol.* **105:** 567–571, 1995.
21. Sambrook J, Fritsch EF and Maniatis T, *Molecular cloning, a laboratory manual*, 2nd ed. Cold Spring Harbour Laboratory Press, New York, 1989.
22. Cohen T, Muller RM, Tomita Y and Shibahara S, cDNA encoding human tyrosinase-related protein. *Nucleic Acid Res.* **18:** 2807–2808,1990.
23. Kwon BS, Haq AK, Pomerantz SH and Halaban R, Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. *Proc. Natl. Acad. Sci. USA* **84:** 7473–7477, 1987.
24. Kwon BS, Chintamaneni C, Kozak CA, Copeland NG, Gilbert DJ, Jenkins N, Barton D, Francke U, Kobayashi Y and Kim YY, A melanocyte-specific gene pmel17, maps near the silver coat color locus on mouse chromosome 10 and is in a syntenic region on human chromosome 12. *Proc. Natl. Acad. Sci. USA* **88:** 9228–9232, 1991.
25. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren J, Bokesch H, Kenney S and Boyd M, New colorimetric cytotoxicity assay for anti-cancer drug screening. *J. Natl. Cancer Inst.* **82:** 1107–1112,1990.
26. Watters D, Beamish H, Marshall K, Gardiner R, Seymour G and Lavin M, Accumulation of HL-60 leukemia cells in G2/M and inhibition of cytokinesis caused by two marine compounds, bistratene A and cycloxazoline. *Cancer Chemother. and Pharmacol.* **33:** 399–409, 1994.
27. Watters DJ, Michael J, Hemphill J, Hamilton SE, Lavin MF and Pettit GR, A novel compound causing changes in protein phosphorylation patterns in human leukemia cells. *J. Cell. Biochem.* **49:** 417–424, 1992.
28. Beretta L, Dobransky T and Sobel A, Multiple phosphorylation of stathmin, *J. Biol. Chem.* **268:** 20076–20084, 1993.
29. Watters D, Garrone B, Gobert G, Williams S, Gardiner R and Lavin M, Bistratene A causes phosphorylation of talin and redistribution of actin microfilaments in fibroblasts: possible role for PKC-δ. *Exp. Cell Res.* **229:** 327–335, 1996.
30. Takahashi H and Parsons PG, *In vitro* phenotypic alteration of human melanoma cells induced by differentiating agents: heterogeneous effects on cellular growth and morphology, enzymatic activity and antigenic expression. *Pigment Cell Res.* **3:** 223–232, 1990.
31. Vijayasaradhi S, Doskoch PM and Houghton AN, Biosynthesis and intracellular movement of the melanoma membrane glycoprotein gp75, the human *b (brown)* locus product. *Exp. Cell Res.* **196:** 233–240, 1991.
32. Abdel-Malek Z, Swope V, Smalara D, Babcock G, Dawes S, and Nordlund J, Analysis of the UV-induced melanogenesis and growth arrest of human melanocytes. *Pigment Cell Res.* **7:** 326–332, 1994.
33. Winder AJ and Harris H, Induction of tyrosinase in human melanoma cells by L-tyrosine phosphate and cytochalasin D. *Exp. Cell. Res.* **199:** 248–254, 1992.
34. Thody AJ and Burchill SA, Tyrosinase and the regulation of coat color changes in C3H-HeAvy mice. *Pigment Cell Res.* **5:** 335–339, 1992.
35. Burchill, SA, Bennett DC, Holmes A, and Thody AJ, Tyrosinase expression and melanogenesis in melanotic and amelanotic B16 mouse melanoma cells. *Pathobiology* **9:** 335–339, 1991.
36. Sabath DE, Broome HE and Prystowsky MB, Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. *Gene* **91:** 185–191, 1990.
37. Desprez PY, Poujol D and Saez S, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, E.C, 1.2.1.12.) gene expression in two malignant human mammary epithelial cell lines: BT-20 and MCF-7. Regulation of gene expression by 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3). *Cancer Lett.* **64:** 219–224, 1992.
38. Arita Y, O'Driscoll KR and Weinstein IB, Growth of human melanocyte cultures supported by 12-O-tetradecanoylphorbol-13-acetate is mediated through protein kinase C activation. *Cancer Res.* **52:** 4514–4521, 1992.
39. Halaban R, Ghosh S, Duray P, Kirkwood JM and Lerner AB, Human melanocytes cultured from nevi and melanomas. *J. Invest. Dermatol.* **87:** 95–101, 1986.
40. Herlyn M, Clark WH, Rodeck U, Mancianti ML, Jambrosic J and Koprowski H, Biology of tumor progression in human melanocytes. *Lab. Invest.* **56:** 461–474 1987.
41. Becker D, Beebe SJ and Herlyn M, Differential expression of protein kinase C and cAMP-dependent protein kinase in normal human melanocytes and malignant melanomas. *Oncogene* **5:** 1133–1139, 1990.
42. Coppock DL, Tansey JB and Nathanson L, 12-O-tetradecanoylphorbol 13-acetate induces transient cell cycle arrest in G1 and G2 in metastatic melanoma cells: inhibition of phosphorylation of p34 cdc2. *Cell Growth and Differ.* **3:** 485–494, 1992.
43. Arita Y, O'Driscoll KR and Weinstein IB, Growth Inhibition of human melanoma-derived cells by 12-O-tetradecanoyl phorbol 13-acetate. *Int. J. Cancer* **56:** 229–235, 1994.
44. Dekker LV and Parker PJ, Protein kinase C-a question of specificity. *Trends Biochem. Sci.* **19:** 73–77, 1994.
45. Yamanishi DT, Graham M, Buckmeier JA and Meyskens Jr FL, The differential expression of protein kinase C genes in normal human neonatal melanocytes and metastatic melanomas. *Carcinogenesis* **12:** 105–109, 1991.
46. Mischak H, Goodnight JA, Kolch W, Martiny-Baron G, Schaechtle C, Kazanietz MG, Blumberg PM, Pierce JH and

Mushinski JF, Overexpression of protein kinase C-δ and -ϵ in NIH3T3 cells induces opposite effects on growth, morphology, anchorage dependence and tumourigenicity. *J. Biol. Chem.* **268:** 6090–6096, 1993.
47. Assender JW, Kontny E and Fredholm BB, Expression of protein kinase C isoforms in smooth muscle cells in various states of differentiation. *FEBS Lett.* **342:** 76–80, 1994.
48. Mischak H, Pierce JH, Goodnight J, Kazanietz MG, Blumberg PM and Mushinski JF, Phorbol ester-induced myeloid differentiation is mediated by protein kinase C-alpha and -delta and not by protein kinase C-beta II, -epsilon, -zeta, and -eta. *J. Biol. Chem.* **268:** 20110–20115, 1993.
49. Belmont L, Mitchison T and Deacon HW, Catastrophic revelations about Op18/stathmin. *Trends Biochem. Sci.* **21:** 197–198, 1996.
50. Larsson N, Melander H, Marklund U, Osterman O and Gullberg M, G2/M transition requires multisite phosphorylation of oncoprotein 18 by two distinct protein kinase systems. *J. Biol. Chem.* **270:** 14175–14183, 1995.
51. Melander H, Marklund U, Larsson N and Chatila TA and Gullberg M, Regulation of microtubule dynamics by Ca^{2+}/calmodulin-dependent kinase IV/Gr-dependent phosphorylation of oncoprotein 18. *Mol Cell Biol* **17:** 3459–3467, 1997.
52. Belmont LD and Mitchison TJ, Identification of a protein that interacts with tubulin dimers and increases the catastrophe rate of microtubules. *Cell* **84:** 623–631, 1996.
53. Marklund U, Larsson N, Gradin MH, Brattsand G and Gullberg M, Oncoprotein 18 is a phosphorylation-responsive regulator of microtubule dynamics. *EMBO J.* **15:** 5290–5298, 1996.
54. Di-Paolo G, Pellier V, Catsicas M, Antonsson B, Catsicas S and Grenningloh G, The phosphorylation of stathmin is essential for nerve growth factor-stimulated differentiation. *J. Cell Biol.* **133:** 1383–1390, 1996.