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Protein kinase C β expression in melanoma cells and melanocytes: differential expression correlates with biological responses to 12-O-tetradecanoylphorbol 13-acetate

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Abstract. Normal human melanocytes require 12-O-tetradecanoylphorbol 13-acetate (TPA) for prolonged growth in vitro. In contrast, the growth of human malignant melanoma cells is often inhibited by TPA. In this study, we have confirmed and extended these observations. Since protein kinase C (PKC) is an important mediator of the effects of TPA, we have investigated the nature of this differential growth response by examining PKC expression and activity in primary cultures of human neonatal melanocytes and metastatic melanoma cell strains. PKC, when measured by immunoreactivity or a functional assay, was found to be more abundant in melanoma cells than in melanocytes. When specific isotypes were examined by Northern analysis, PKC-α and -ϵ were expressed in both melanocytes and melanoma. PKC-β was expressed in melanocytes, but was undetectable by Northern analysis in 10 out of 11 melanoma cell strains. Southern analysis revealed that no gross deletions or rearrangements of the PKC-β gene had occurred. These data suggest that down-regulation of the PKC-β gene occurs frequently during the process of transformation of melanocytes. Furthermore, differential expression of PKC isotypes may explain the different effects of TPA on melanocyte and melanoma cell growth.

Key words: Transformation – Regulation – Protein kinase C

Introduction

The progression of malignant melanoma from melanocytes through precursor lesions (dysplastic nevi) to metastatic tumors is a multistep process, and distinct stages have been identified and characterized (Elder 1987). Our basic understanding of melanoma has been hampered by a lack of information on normal melanocytes and the mechanisms by which these cells become transformed. With refinement of the in vitro culturing of melanocytes over the past few years, the biological, immunological, and genetic characteristics of these cells are being investigated (Herlyn et al. 1987; Houghton et al. 1987).

The phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA) is both mitogenic for normal melanocytes and required for their sustained growth in culture (Eisinger and Marko 1982; Eisinger et al. 1983). Paradoxically, metastatic melanoma cells are often inhibited by this same compound (Halaban et al. 1986). Within the cell, the effects of the tumor-promoting phorbol esters are mediated by intracellular receptor proteins; the major one being protein kinase C (PKC). PKC is known to play an important role in cellular proliferation and may also be involved in the process of transformation (Parker et al. 1986; Persons et al. 1988; Krauss et al. 1989; Megidish and Mazurek 1989; Choi et al. 1990). It has been implicated in the in vitro transformation processes caused by multiple oncogenes (Krauss et al. 1990). Activation of PKC results in increased transcription of *fos* and *jun*, two proto-oncogenes the nuclear protein products of which form a heterodimer that is part of the AP-1 complex. This complex has been shown to bind to the regulatory element of promoter regions of genes that are induced by treatment of cells with TPA (Distel and Spiegelmann 1990; Vogt and Bos 1990).

Molecular cloning and biochemical analysis of PKC indicate there is a family of PKC genes. At least seven different isotypes have been isolated from bovine and human brain cDNA libraries, which map to different chromosomes (Parker et al. 1989; Farago and Nishizuka 1990). Slightly different modes of activation and substrate specificities have been described (Parker et al. 1989), and brain is the only tissue in which all of the isotypes have been reported (Kikkawa et al. 1988). It has been proposed that the high degree of conservation and the distinct distribution patterns in the brain and peripheral tissue indicate that different isotypes have different functions in regulation and substrate specificity (Parker et al. 1989; Farago and Nishizuka 1990).

In this study we have investigated the effect of TPA on the growth, PKC expression, and PKC activity of melanocytes and melanoma cell strains.

Materials and methods

Reagents. Hamm's F10 and MCDB 153 medium were purchased from Irvine Scientific (Santa Ana, Calif.), penicillin and streptomycin were purchased from Gibco (Grand Island, N.Y.), and bovine pituitary extract was purchased from Clonetics (San Diegeo, Calif.). Radio-labelled nucleotides and amino acids were obtained from New England Nuclear (Boston, Mass.) and ICN (Costa Mesa, Calif.). M-15 medium was provided by A. Lebowitz (Meltzer et al. 1991). All other chemicals were purchased from Sigma (St. Louis, Mo.).

Cells and tritiated-thymidine-uptake assay. Melanocytes were isolated from newborn foreskin and cultured using a combination of the procedures developed by Eisinger and Marko (1982) and Halaban and Alfano (1984). Culture conditions have been described in detail by Halaban and colleagues (Halaban et al. 1987) and Herlyn et al. (1988). The melanocytes used for these studies were isolated from newborn foreskin taken during routine circumcisions. The cells were maintained in MCDB 153 medium containing 2% fetal bovine serum, penicillin (100 U/ml), streptomycin (0.1 mg/ml), isobutylmethylxanthine (0.1 m*M*), bovine pituitary extract (70 µg/ml), and TPA (10 ng/ml).

Melanoma cell strains (C81-61, C81-46C, C81-46A, C83-2C, K457, H383, S1273, L2022, and G2306) were developed from patient biopsy samples and analyzed at passage 10 or less, as previously described (Thomson and Meyskens 1982). Two cell strains, C81-46A and C81-46C, were isolated from two different tumors from the same patient. Cell lines 1675 and 1676 were obtained from ATCC. C81-61, C81-46C, C81-46A, C83-2C, 1675 and 1676 were cultured in medium consisting of Hamm's F10 medium, penicillin (100 U/ml), streptomycin (0.1-mg/ml) and 5% fetal bovine serum/5% newborn calf serum. Melanoma cell strains, K383, H454, S1273, L2022, and G2306 were cultured in M-15 medium containing 5% fetal calf serum (Meltzer et al. 1991). All of the melanoma cell strains used for these experiments, with the exception of 1675 and 1676, were developed at the Arizona Cancer Center and were analyzed at low passage numbers. The two cell lines, 1675 and 1676, were beyond passage 35.

The effect of TPA on uptake of [³H]thymidine was evaluated by incubating 5× 10⁴ cells in a 96-well microtiter plate with and without TPA (10 ng/ml) in medium containing 1% fetal calf serum for 72 h. [³H]Thymidine (0.5 µCi/well; 90 Ci/mmol, NEN, Boston, Mass.) was added to each well for the last 16 h of the incubation. The cells were harvested onto glass-fiber filters using a cell harvester (PhD Harvester, Cambridge Research Inc., Valley Stream, N.Y.). The filters were placed in scintillation fluid (Cytoscint-ES; ICN, Costa Mesa, Calif.) and the amount of incorporated [³H]thymidine was determined by liquid scintillation counting (LS5000TD, Beckman, Fullerton, Calif.).

Protein kinase C assay. PKC activity was assayed by measuring the transfer of phosphate from [³²P]ATP onto histone H1. Cytosol and detergent-solubilized membrane fractions of the cells were prepared as described by Thomas et al. (1987). The standard reaction mixture contained 20 m*M* Tris/HCl (pH 7.5), 0.75 m*M* calcium chloride, 10 m*M* magnesium acetate, 100 m*M* [³²P]ATP (120 cpm/pmol), 25 µg histone H1, 50 µg/ml leupeptin, with or without phosphatidylserine (24 µg) and diolein (2 µg). After incubation at 30° C for 3 min, the reaction was terminated by the addition of ice-cold 25% trichloroacetic acid (Cl₃A_cOH). The precipitate was collected onto 0.45-µm nitrocellulose filters. The reaction tubes were washed twice with ice-cold 5% Cl₃A_cOH and the contents were applied to filters. The filters were then washed with cold 5% Cl₃A_cOH, dissolved in Cytoscint-ES (ICN Biomedical, Costa Mesa, Calif.) scintillation fluid, and counted for radioactivity in a liquid scintillation counter (LS5000TD, Beckman). PKC activity was expressed as pmol ³²P incorporated (3 min)⁻¹ (30 µg protein)⁻¹. This calculation was corrected for ³²P incorporation by histone in the absence of added phospholipid.

Evaluation of PKC expression by flow cytometry. Cells were stained using an indirect immunofluorescence procedure and analyzed using a fluorescence-activated cell sorter (FACS SCAN, Becton Dickinson). Briefly, the cells were fixed with 50% methanol/50% phosphate-buffered saline (PBS) for 10 min, washed several times with PBS and then incubated with an anti-PKC mouse monoclonal antibody, which recognizes both α and β isotypes (clone MC5; Amersham, Arlington Heights, Ill.) or a rabbit polyclonal anti-PKC-ß antibody (Gibco/BRL, Grand Island, N.Y.) at the appropriate dilution. Recognition of antigenic determinants was revealed by incubation of the cells with a secondary antibody, fluoresceinated goat anti-(mouse IgG) [H&L F(ab')₂; Caltag, So. San Francisco, Calif.] or fluoresceinated swine-anti-(rabbit immunoglobulins) (Dakopats, Santa Barbara, Calif.). Fluorescence of the cells was quantitatively analyzed with a FACS SCAN and Consort 30 software. The FACS SCAN utilizes a 488-nm argon laser for fluorescene excitation and a 530-nm filter set for green fluorescence detection. The cells were 80% confluent before harvesting and analysis.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated using a modification of the guanidinium isothiocyanate method described by Chirgwin et al. (1979). Cells were lysed in a 4 *M* guanidinium isothiocyanate, 25 m*M* EDTA, 0.5% sodium *N*-dodecylsarcosine solution. CsCl was added to the lysate and the homogenate was sheared and layered onto a 5.7 *M* cesium chloride cushion. The RNA pellet was extracted with phenol/chloroform, ethanol-precipitated, and resuspended in sterile water. Northern blots were prepared according to Fourney et al. (1988). Samples were electrophoresed through a 6% formaldehyde/agarose gel and transferred to a nylon filter (Nytran, Schleicher and Schüll, Keene, NH). The filters werel prehybridized for 4 h at 42° C in 50% formamide, 5× SSPE (0.18 *M* NaCl, 10 m*M* sodium phosphate pH 6.7, 1 m*M* EDTA), 0.1% sodium dodecyl sulfate (SDS), 0.5× Denhardt's solution, and 2 mg/ml salmon sperm DNA. Hybridization of the filter with radiolabelled probes was performed in hybridization solution, which was the same as the prehybridization solution with the addition of 5% dextran sulfate.

The PKC cDNA probes and 18S cDNA probe were ³²P-labelled by the method of Feinsberg and Vogelstein (Freinberg and Vogelstein 1983) using a random-priming kit (Promega, Madison, Wis.). The steady-state levels of RNA transcripts were quantified with a scanning densitometer (GS 300, Hoefer, San Francisco, Calif.). For all Northern analyses, the amount of RNA loaded in each lane was normalized by the hybridization with the 18S cDNA probe, so that differences in RNA loading and transfer to the membrane could be corrected for quantitative analysis. RNA standards (0.24- to 9.5-kb RNA ladder; BRL, Gaithersburg, Md.) were used to determine transcript sizes. Rat brain RNA was included on the Northern blots as a positive control (data not included).

DNA isolaltion and Southern analysis. High-molecular-mass DNA was isolated from cells as described by Davis et al. (Davis et al. 1986). Briefly, monolayers were harvested and washed twice in PBS. Cell pellets were resuspended in lysis buffer [0.2 *M* TRIS, 0.1 *M* EDTA, 0.5% SDS, proteinase K (50 µg/ml; Sigma), RNase A (10 µg/ml] and incubated at 37° C overnight with gentle agitation. Sodium chloride was added to a concentration of 0.5 *M*. The preparations were extracted twice with phenol and then with chloroform/isoamyl alcohol (24 : 1). DNA was precipitated with ethanol (2 volumes) and resuspended in 10 m*M* TRIS, 1 m*M* EDTA (pH 8.0). Samples were quantified spectrophotometrically.

Samples of 10–15 µg DNA from each cell strain or line were digested to completion with restriction enzymes *Pvu*II, *Hind*III, *Bam*H1, or *Eco*R1 and size-fractionated by electrophoresis through a 0.9% agarose gel prepared with 1× TAE buffer (Davis et al. 1986). Gels were stained with ethidium bromide to document DNA size marker migration. The gels were soaked in 1.5 *M* NaCl and 0.5 *M* NaOH and subsequently neutralized in a solution containing 1.5 *M* NaCl, 0.5 *M* TRIS/HCl and 1 m*M* EDTA. Transfer to nylon membranes was by capillary action overnight using 10× standard saline citrate. Filters were processed as described for Northern blots above.

Probes. The PKC-α, -β, and -γ cDNAs were obtained from Genentech (San Bruno, Calif.) (Coussens et al. 1986). The PKC-α probe consisted of a 1.3 × 10³-base (1.3-kb) *Eco*RI restriction fragment from the plas-

mid, phPKC-α7; the PKC-β-probe consisted of a 0.9-kb EcoRI fragment of the phPKC-B1-15-EcoR12 plasmid, and the PKC-γ probe included a 1.0-kb BamHI restriction fragment from the phPKC-γ6 plasmid. A second PKC-β plasmid, pMV7-PKC-β1, kindly provided by Dr. Robert Krauss, was used to isolate cDNA fragments specific for the 5' end of the PKC gene, the constant region of the gene, and a PKC-β2-specific fragment (see Fig. 5 for a description of the PKC-β probes; Housey et al. 1988). The PKC-ε probe provided by Dr. J. L. Knopf (Knopf et al. 1986) was a 1.5-kb PstI/EcoRI restriction fragment from the pmt-PKC-ε plasmid. A 5.6-kb EcoRI restriction fragment from the plasmid, pB, was used to detect 18S rRNA. The 18S cDNA probe was kindly provided by Dr. J. E. Sylvester (Gonzalez et al. 1988).

Results

Biological response of melanocytes and melanomas to TPA

Normal melanocyte cultures have a limited capacity for replication in vitro unless they are grown in TPA-supplemented media. Cell numbers begin to decrease within 3–4 days after removal of the TPA from the cultures. Cloned melanoma cell strains (C81-46A, C81-46C, C81-61, C83-2C, K457) proliferate in serum-supplemented media. In contrast to the melanocytes, the growth of melanoma cells is often inhibited by TPA. Figure 1 illustrates the effect of 10 ng/ml TPA on the uptake of [³H]thymidine by melanocytes and melanoma cell strains. With melanocytes we observed a 21-fold increase in [³H]thymidine incorporation following incubation with TPA for 72 h. The melanoma strains varied in their sensitivity to TPA. DNA synthesis in C81-46C was inhibited 45%; C81-46A showed a 59% recuction in [³H]thymidine uptake; and incorporation in C81-61 was reduced by 49%. Cell strains C83-2C and K457 were least sensitive to TPA, with only an 18% decrease in [³H]thymidine uptake. On the basis of trypan blue exclusion the inhibition was cytostatic rather than cytotoxic (unpublished observations). Because of the observed differences in growth of melanomas and melanocytes

in response to TPA, we next investigated PKC, an important mediator of TPA activity.

PKC activity in melanocytes and melanoma cells

PKC activity of cytosolic and membrane fractions was measured in cell lysates from normal melanocytes and melanoma cells, before and after treatment with TPA (Table 1). Baseline levels were measured 24 h after TPA (melanocytes) or serum (melanoma) had been removed from the growth medium.
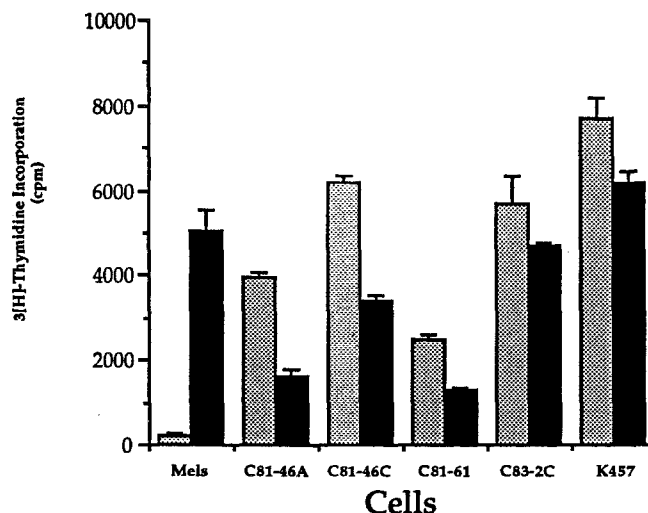

Fig. 1. 12-O-tetradecanoylphorbol 13-acetate (TPA) stimulates the uptake of [³H]thymidine by melanocytes (Mels) and inhibits [³H]thymidine incorporation by melanoma cells. Melanoma cells (5 × 10⁴) and melanocytes (1 × 10⁵) were cultured in 96-well microculture plates in medium containing 1% fetal calf serum with (solid bars) or without (hatched bars) 10 ng/ml TPA for 72 h. For the last 16 h of the incubation the cultures were pulsed with 0.5 μCi/well [³H]thymidine. The boxes represent the means ± SE of triplicate cultures. Representative results are shown from two experiments

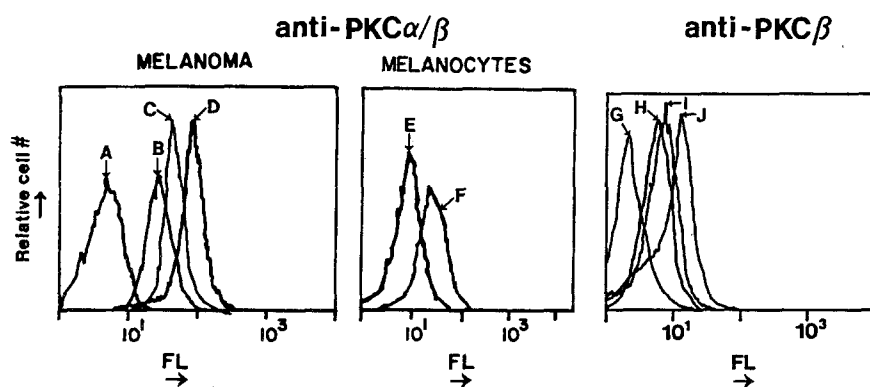**Fig. 2.** Determination of protein kinase C (PKC) expression by flow cytometry. Approximately 1 × 10⁶ cells of each cell strain were permeabilized and then incubated sequentially with an anti-PKC antibody and a fluorescein-conjugated secondary antibody. The cells were analyzed using a FACS SCAN (Becton Dickinson). In the first two panels, the primary antibody was anti-PKC-α/β. A, E, The level of fluorescence detected when the melanoma cells, C81-46A (A) and melanocytes (E) were incubated with only the secondary fluorescein-conjugated goat anti-(mouse Ig)antibody. Background fluorescence for the other melanoma cell strains was not different from A. PKC-α/β was detected in melanoma cell strains, C81-61 (B), C81-46A (C), C81-46C (D), and melanocytes (F). In the third panel, the cells were stained with a rabbit anti-(PKC-ß peptide)polyclonal antibody. Data are shown for C81-61 (H), K457 (I), and melanocytes (J). Curve G shows the level of background fluorescence when melanoma cell strain C81-61 is incubated with the fluorescein-conjugated swine anti-(rabbit Ig)antibody alone. The backgrounds for K457 and melanocytes are nearly identical to that of C81-61

Table 1. Protein kinase C (PKC) activities measured in the cytosol and membrane fractions of normal melanocytes and melanoma cells [a]

Incubation medium [b]	PKC activity [pmol ^{32}P (3 min)$^{-1}$ (30 µg protein)$^{-1}$]							
	Melanocytes		Melanoma cells					
			C81-61		C81-46C		C83-2CY	
	Cyt	Mem	Cyt	Mem	Cyt	Mem	Cyt	Mem
Basal	38	58	90	30	188	83	310	95
TPA	45	98	144	480	60	390	87	510

[a] Protein kinase C activity was calculated as the difference between the activities in the presence and absence of phosphatidylserine/diacylglycerol. Each number represents the mean of triplicate samples. All standard errors were less than 10%. Representative results of two experiments are shown

[b] Basal medium: melanocyte, 0.5%; fetal calf serum; no 12-O-tetradecanoylphorbol 13-acetate (TPA); melanoma, no serum or TPA. TPA medium: incubation in presence of TPA for 1 h

Cytosolic PKC activity was significantly greater in the three melanoma cell strains (90–310 pmol) than in the melanocytes (38 pmol). Membrane-bound PKC in the melanocytes (58 pmol), however, was within the range observed for the melanomas (30–188 pmol). Upon treatment with TPA (10 ng/ml) for 1 h, there was an increase in measurable PKC activity and a translocation of PKC from the cytosol to the membrane in both the melanocytes and melanoma cells. The degree of translocation was greater for the melanoma cells than the melanocytes.

PKC protein

The level of immunoreactive PKC protein in the melanomas and melanocytes was analyzed by flow cytometry (Fig. 2). The cells were cultured in growth medium and examined for PKC protein expression when the cultures reached 80% confluence. Permeabilized cells were incubated with either a monoclonal anti-PKC-α/β or a polyclonal PKC-β-peptide-specific primary antibody. A fluorescein-conjugated secondary antibody was used for detection. In the melanocytes (Fig. 2F), the level of PKC-α/β protein detected was similar to the lowest level of PKC-α/β protein observed in the melanomas (C81-61, Fig. 2B). The background fluorescence in melanocytes however, was higher (Fig. 2E) than in the melanoma cell strains with the goat anti-(mouse Ig)secondary antibody. In the melanoma cell strains C81-46A and C81-46C (Fig. 2C, D), the increased intensity of fluorescence detected on positively stained cells indicated the presence of more immunoreactive PKC-α/β. With the PKC-β-specific antibody, the melanocytes (Fig. 2J) had the greatest level of immunoreactivity, while C81-61 (Fig. 2H) and K457 (Fig. 2I) had a lesser quantity of protein that was detectable above background (Fig. 2G). The protein detected in C-81-61 and K-457 may represent non-specific binding of the antibody.

Gene expression

The steady-state mRNA transcript levels and regulation of different PKC isotypes were investigated. Melanocytes and
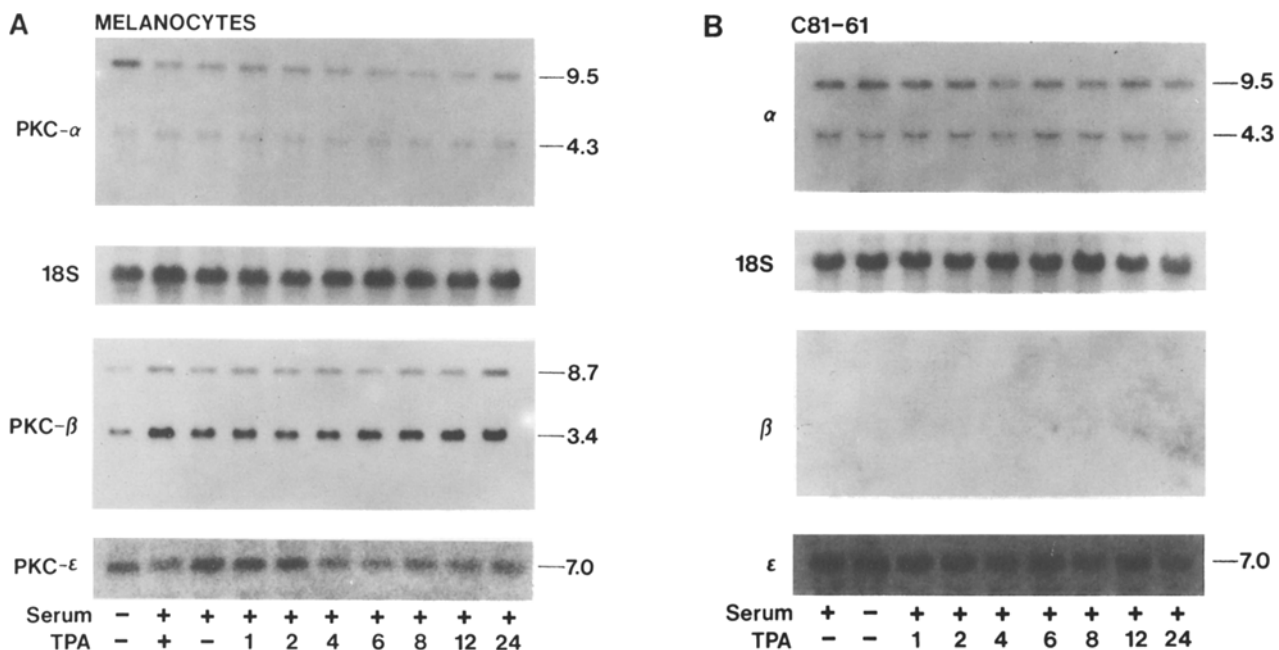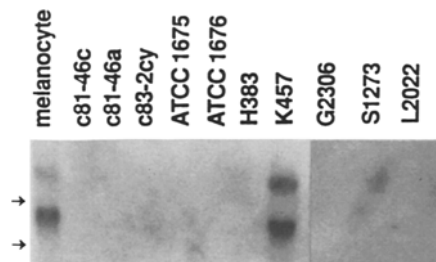

Fig. 3A, B. Northern analysis of PKC isotype expression and regulation by TPA. Total RNA was isolated from cultures of melanocytes (**A**) and melanoma cell strains (**B**; only C81-61 is presented) that were grown with and without serum (5% fetal bovine serum) and/or TPA (10 ng/ml). **A** Melanocyte cultures were grown in the absence of TPA for 24 h prior to the addition of TPA (10 ng/ml) for 1, 2, 4, 6, 8, 12, and 24 h. **B** TPA was added to flasks of melanoma cells for the times indicated above. Total RNA (10 µg/lane) was used to prepare Northern blots. The filters were hybridized with ^{32}P-labelled PKC-α, -β, and -ϵ cDNA probes (Knopf et al. 1986; Housey et al. 1988); see Materials and methods). An 18S cDNA probe was used to standardized the blots. Bands were measured with a scanning densitometer and the values generated were used to correct for loading or transferring error when comparing the PKC transcripts levels

melanoma cells were cultured in the presence or absence of TPA and Northern blot analysis was carried out on total RNA (Fig. 3A, B, Fig. 4). The resulting filters were hybridized with ^{32}P-labelled cDNA probes of PKC-α, -β, -γ, and -ϵ. Autoradiographs of the Northern blots (Fig. 3A) showed expression of three isotypes, α, β, and ϵ, in TPA-treated and untreated melanocytes. Two transcript sizes were observed for PKC-α (9.5 kb and 4.3 kb) and PKC-β1 (8.7 kb and 3.4 kb), and one size transcript for PKC-ϵ (7.0 kb) (Fig. 3A). In three of the melanoma cell strains, C81-61 (Fig. 3B), C81-46A, and C81-46C (data not shown), both PKC-α and -ϵ were detected; in melanoma C83-2C only PKC-ϵ was detected (Yamanishi et al. 1991). PKC-β transcripts were not observed in the TPA-treated nor in the untreated melanoma strains (Fig. 3B); PKC-γ was not observed in either the melanoma cells or the melanocytes (data not shown). The sizes of the PKC transcripts we observed are similar to those reported previously (Coussens et al. 1986).

Two forms of PKC-β exist, which arise from alternative splicing of the terminal exon (see Fig. 5 for details). To confirm and augment the observation that PKC-β was expressed in melanocytes and not melanoma, we examined total RNA from additional melanoma cells with both PKC-β1- and PKC-β2-specific probes. PKC-β expression was absent in 10 out of 11 melanomas (Figs. 3 B, 4). PKC-β1 was detected in the melanocytes and in one of the melanoma cell strains, K457 (Fig. 4). PKC-β2 was not detectable in either the melanocytes or melanoma cell strains (data not shown).

The effects of TPA on the level of expression of PKC-α, -β, and -ϵ was also examined to determine whether alterations in the expression of these genes may help to explain the differential growth response of melanoma and melanocytes to TPA. Northern blots of RNA isolated from melanocytes and melanoma cells, C81-46C, C81-46A, C81-61, and C832C, exposed to TPA (10 ng/ml) for various lengths of time, were hybridized with the PKC-α, -β, -ϵ cDNA probe and an 18S cDNA probe as a control for loading amounts (Fig. 3A, B). The autoradiographs were then screened on a scanning densitometer. For melanocytes (Fig. 3A), we observed no significant effect of TPA or serum on α or ϵ transcript levels. PKC-β1 expression was modestly induced in response to TPA at 12 h (20%–60%). After 24 h, the 8.7-kb transcript was increased twofold. Among the melanoma cell strains, the expression of PKC-α and -ϵ in response to TPA

was highly variable. The following patterns emerged from several experiments. (a) PKC-α expression was depressed by TPA (20%–50%) and increased by serum (10%–50%) in C81-61 (Fig. 3B) and C81-46A; whereas expression of both α transcripts was increased two- to sixfold by TPA and decreased sevenfold by serum in C81-46C (data not presented). (b) ϵ transcripts were either depressed or unchanged by exposure to TPA for C81-61 (0%; Fig. 3B), C81-46C (31%), and C81-46A (43%).

(c) In the cell line C83-2C, which expressed PKC-ϵ but did not express PKC-α, PKC-ϵ was increased (50%–60%) by TPA exposure.

These observations of the regulation of PKC-α and -ϵ RNA transcript expression indicate considerable heterogeneity of response to TPA with no distinct pattern differences between the melanocytes and melanomas.
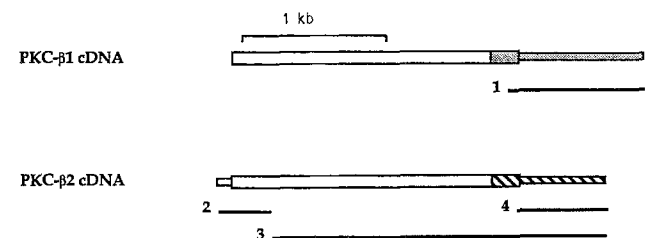
Southern analysis

Southern analysis was used to determine whether PKC-β gene deletion or rearrangement could account for the loss of PKC-β expression. DNA samples from the 11 melanoma cell
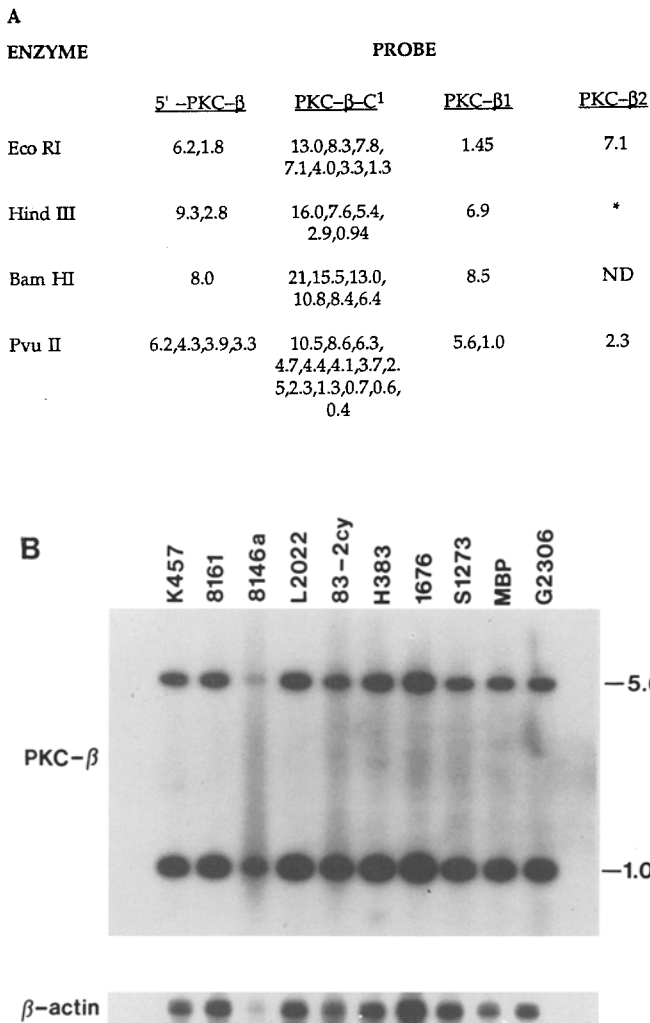
A. Alternative Splicing of the Terminal Exons of PKC-β

B. PKC-β Probes
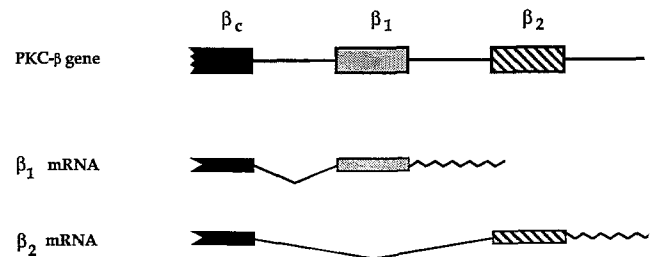
Fig. 5A, B. PKC-β1 and -β2. **A** A schematic diagram of the alternatively spliced terminal exon of PKC-β [modified from Coussens et al. (1987) and Kubo et al. (1987)]. The nomenclature is derived from Coussens et al. (1987). **B** PKC-β probes used for Northern and Southern hybridizations include: *1*, the β1-specific probe is a 891-base-pair (bp) *Eco*RI fragment from phPKC-B1-15 (a generous gift from Genentech); *2*, the 5' probe is a 327-bp *Eco*RI fragment from pMV7-PKC (a generous gift from I. Bernard Weinstein); *3*, the common β probe is a 2.2-kb *Eco*RI fragment from pMV7-PKC; *4*, the β2-specific probe is a 600-bp *Acc*I fragment of pMV7-PKC. *Wide boxes* correspond to the open-reading frame and *narrow boxes* correspond to the untranslated regions. *Filled portions* correspond to β1 (*stippled*) and β2 (*cross-hatched*) specific regions

Fig. 4. Northern analysis of PKC-β1 expression in normal melanocytes and in 11 malignant melanoma cell strains and lines. Samples of 10 µg total RNA were used. The blots were hybridized with ^{32}P-labelled PKC-β1 cDNA probes (see Fig. 5 for a description). *Arrows* indicate the positions of 28S and 18S ribosomal RNA. Four independent isolates of RNA from melanocytes and each melanoma gave identical results

A

ENZYME

		PROBE		
	5'-PKC-β	PKC-β-C[1]	PKC-β1	PKC-β2
Eco RI	6.2,1.8	13.0,8.3,7.8, 7.1,4.0,3.3,1.3	1.45	7.1
Hind III	9.3,2.8	16.0,7.6,5.4, 2.9,0.94	6.9	*
Bam HI	8.0	21,15.5,13.0, 10.8,8.4,6.4	8.5	ND
Pvu II	6.2,4.3,3.9,3.3	10.5,8.6,6.3, 4.7,4.4,4.1,3.7,2. 5,2.3,1.3,0.7,0.6, 0.4	5.6,1.0	2.3

B

Fig. 6A, B. Summary of Southern analysis of the PKC-β gene in human melanoma cell strains and lines. **A** Restriction fragments of the human PKC-β gene. A 10-μg sample of genomic DNA was digested with four restriction enzymes, *Eco*RI, *Hind*III, *Bam*HI, and *Pvu*II. Southern blots were hybridized with the four PKC-β probes that represent the 3'region, the β common region, a PKC-β1-specific region and a β2-specific region (described in Fig. 5). The major fragments that were observed are listed. *, Fragments were too small to be detected; ND, not done. **B** Representative Southern blot. Melanoma genomic DNA digested with *Pvu*II and probed with the PKC-β1-specific cDNA fragment. Eight samples of DNA from malignant melanoma and DNA isolated from peripheral blood leukocytes from a normal individual (M.B.P.) are shown

strains and lines and from white blood cells from a normal individual were digested with four restriction enzymes, *Eco*RI, *Hind*III, *Bam*HI, and *Pvu*II (Fig. 6). Southern blots prepared with the digested DNAs were probed with PKC-β cDNA fragments that recognized the 5' end of the gene, a constant region found in both PKC-β1 and PKC-β2, a PKC-β1-specific fragment, and a PKC-β2-specific fragment (Fig. 5A, B). The fragment sizes observed with each probe and enzyme are summarized in Fig. 6. The gene was present in all 11 of the melanomas. There were no gross rearrangements or deletions in 10 of the cell strains or lines. In 81-61, the normal 8.0-kb *Bam*HI fragment detected with the 5' PKC probe was present and accompanied by additional fragments of 4.6 kb and 2.9 kb.

Discussion

Cultivation of primary human melanocytes in vitro has become possible with the discovery that TPA is a potent mitogen for these cells (Eisinger and Marko 1982; Eisinger et al. 1983). TPA has been shown in a variety of in vivo and in vitro systems to function as a tumor promoter (Diamond et al. 1980; Yuspa and Poirier 1988). While it has not been demonstrated that phorbol ester tumor promoters contribute to the transformation of melanocytes, the mitogenic activity of TPA in these cells suggests that TPA-dependent pathways are active. We and others (Halaban et al. 1986) have observed markedly different effects of TPA in melanoma. In contrast to melanocytes, TPA was not mitogenic for melanoma cells. In fact, of the five cell strains described in Fig. 1, the incorporation of [3H]tyhmidine was inhibited 45%–59% by TPA in three cell strains (C81-46A, C81-46C, and C81-61). This pattern of TPA inhibition demonstrated a dose response over a range of TPA concentrations (1–50 ng/ml) tested and the inactive phorbol ester, 4-α-phorbol 12,13-didecanoate, had no effect on the growth of the melanomas and does not sustain growth of the melanocytes (data not presented).

Having defined contrasting growth responses of melanocytes and melanoma cell strains to TPA, we proceeded to investigate the nature of this difference by examining the expression of PKC in these cells. The levels of PKC-α/β protein detected by flow cytometry indicated greater immunoreactive protein in the melanomas than the melanocytes. These results concur with our finding that cytosolic and membrane extracts from the melanoma cells contained more PKC enzymatic activity than extracts from normal melanocytes. Our flow-cytometry results with an anti-PKC-β-specific antibody demonstrated more immunoreactive PKC-β protein in melanocytes than in the melanoma cells. Chida and colleagues (Chida et al. 1988) have reported that in a variety of normal and nontransformed cells there are higher levels of PKC activity than in malignant and transformed cells. This group did not directly compare PKC activity in melanoma and melanocytes. Our results, which show lower levels of PKC activity in melanocytes and a higher proportion of membrane-associated PKC relative to cytosolic levels at baseline, must also be interpreted with caution since the melanocytes were grown in media with TPA and calcium for an extended period of time prior to its removal for these experiments. Chronic exposure to TPA has been shown to downregulate PKC activity in other cell types (Chida et al. 1986; Young et al. 1987). Nevertheless, our results are consistent with a growing body of evidence that differential expression of the PKC isotypes may account for the diversity and nature of responses to activation of PKC (Nishizuka 1988; Parker et al. 1989).

We have further explored whether the expression of PKC-α, -β, -γ, and -ε isotypes is different in melanomas compared to normal melanocytes. Contrary to a recent report by Becker and colleagues (Becker et al. 1990), we were able to detect PKC-α, -β1, and -ε transcripts in RNA isolated from primary cultures of newborn human melanocytes. PKC-β2 and PKC-γ were not expressed.

We have previously reported that PKC-α and -ε transcripts were detected in the melanoma cells (Yamanishi et al. 1991). PKC-α was expressed at levels similar to or greater

than those seen in the melanocytes. PKC-ϵ was expressed at similar levels in both melanocytes and melanomas. We have further shown that PKC-α and -ϵ mRNA levels are not significantly affected by TPA. PKC-β1 mRNA was undetectable in all but 1 of 11 melanoma cell strains and lines using Northern analysis. In the melanoma (K457) that expressed PKC-β1 mRNA, the level of protein was significantly below that detected in melanocytes and was only slightly greater than that observed for C81-61, which does not express PKC-β mRNA (Fig. 3B; Yamanishi et al. 1991). These data suggest that K457 may have an alteration in the process of translation of the PKC-β protein or that the protein lacks the antigenic determinant recognized by the anti-PKC-β antibody. Additional studies are underway to evaluate these possibilities.

The differential expression of PKC-ß may provide an important clue for understanding the inhibitory effects of TPA on the growth of melanomas. One possible explanation is that PKC-β is a tumor-suppressor gene. Recently, Choi et al. (1990) have found that their PKC-β1 (corresponding to PKC-β2 in Fig. 5) can function as a tumor suppressor in colon cancer cells. This hypothesis would account for the loss of PKC-β expression in the process of transformation from melanocytes to melanoma. However, it fails to explain why TPA stimulates the growth of melanocytes but is inhibitory for melanoma. A satisfactory model needs to address the three significant observations: (a) PKC-β expression is lost in the melanomas, (b) TPA stimulates melanocyte growth and (c) TPA fails to stimulate and, in some cases, is inhibitory to melanoma cell growth. A model that accounts for all of the data proposes that PKC-β1 functions as a tumor suppressor in its unstimulated state and as a growth stimulator in the presence of TPA. In this model, the inhibitory effects of TPA on the growth of melanoma cells results from an imbalance of the complex interactions amongst different isotypes of PKC other than PKC-β1. An alternative model is that PKC-β1 expression is associated with TPA-regulated growth. In melanocytes, the regulation is positive, whereas in the process of transformation to melanoma, this regulation becomes inhibitory and consequently PKC-β expression is counterselected in the population. A mechanism to account for these different regulatory effects could be the availability of different substrates for phosphorylation or other proteins that interact with PKC-β. A final possibility is that the differential biological effects of TPA are unrelated to the differential expression of PKC-β1. The high frequency of loss of PKC-β expression in melanomas, however, argues against this. Our data are consistent with the idea that the variety of biological responses seen with different cell types in response to TPA or activators of the PKC system is due to complex interactions amongst different isotypes of PKC. We are currently introducing a PKC-β1 expression construct into melanoma cell lines to further evaluate its biological effects.

Gene deletion or rearrangement could account for the loss of PKC-β expression in the melanoma cells. Linnenbach et al. found that the PKC-α gene had been homozygously deleted in one melanoma cell line (Linnenbach et al. 1988). Using Southern analysis with four different probes and four restriction enzymes, we found no alterations of the PKC-β locus in 10 of the 11 melanoma cell strains and lines. In cell strain C81-61, abnormal fragments were detected at the 5' end of

the gene. However, these were only detected with one restriction enzyme (BamH1) and most likely represents a polymorphism of a restriction fragment length. Thus PKC-β gene rearrangement or deletion does not appear to be an important mechanism of loss of PKC-β expression in the malignant transformation of human melanocytes. Although a significant reduction in mRNA stability is a formal possibility to explain these results, we conclude that the observed loss of expression is due to down-regulation of the gene. If down-regulation has occurred, it could be due to the loss of a transactivator, the presence of a transrepressor or a cis mutation in the PKC-β promoter. Currently we are evaluating the human PKC-β promoter to explore these possibilities.

Our findings have potential clinical implications for human melanoma. With the development of high-affinity PKC-β-specific antibodies, loss of PKC-β expression may serve as a marker of melanocyte transformation. Furthermore, pharmacological manipulation of the activity of specific PKC isotypes could lead to the development of novel therapies for malignant melanoma. Consistent with this idea, Schuchter and colleagues (Schuchter et al. 1991) have demonstrated that bryostatin-1, which is an inhibitor of PKC, has an antitumor effect in a mouse melanoma model.

In summary, the differential response of melanocytes and melanoma cell strains to TPA is associated with a differential expression of PKC-β, suggesting that an altered balance of PKC isotypes may be responsible for the biological effects. Characterization of the biological effects of PKC-β and an understanding the mechanism that has led to the loss of PKC-β expression should provide further insights into the process of malignant transformation of human melanocytes.

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