Molecular Biology of the Cell
Vol. 5, 497–509, April 1994

Terminal Differentiation and Senescence in the Human Melanocyte: Repression of Tyrosine-Phosphorylation of the Extracellular Signal-regulated Kinase 2 Selectively Defines the Two Phenotypes

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Submitted November 9, 1993; Accepted March 1, 1994
Monitoring Editor: Joseph Schlessinger

Melanocytes are pigmented cells distributed in humans in several organs like the epidermis, the leptomeninges, the eye, and the inner ear. Epidermal melanocytes, whether derived from adult or neonatal skin, proliferate well in a medium supplemented with phorbol esters and other mitogens before they undergo senescence. Potent cAMP inducers like cholera toxin are also growth promoters for neonatal melanocytes but only transient growth stimulators for cells derived from adults. We used this cellular system to delineate biochemical pathways involved in proliferation and in terminal differentiation. Here we show that after a period of 4–8 wk of sustained proliferation in the presence of cholera toxin, the adult melanocytes became round, flat, and enlarged. These changes were associated with terminal growth and preceded by a five- to sixfold increase in cAMP levels and an 8- to 10-fold increase in melanin content. The simultaneous addition of phorbol esters and cholera toxin did not prevent cells from reaching terminal differentiation. Identified targets for phorbol esters are protein kinase C (PKC) and the mitogen-activated kinases (MAPKs), also called extracellular signal-regulated kinases (ERKs). PKC was found to be similarly regulated in proliferating and in terminally differentiated melanocytes. Proliferating melanocytes in early or late passage showed identical activation of the kinase ERK2. This kinase was rapidly phosphorylated upon phorbol 12-myristate 13-acetate (PMA) addition and specifically accumulated in the nucleus of the cells, whereas in unstimulated cells it had a perinuclear distribution. In contrast, senescent and terminally differentiated cells were unable to phosphorylate tyrosine residues of the ERK2 gene product in spite of presenting normal amounts of ERK2 protein. In addition, ERK2 did not show the nuclear accumulation observed in proliferating melanocytes after PMA activation and remained localized in the perinuclear area. These results demonstrate that senescent and terminally differentiated melanocytes share a common block in a critical pathway thought to integrate multiple intracellular signals transmitted by various second messengers and specifically prevent the continuation of the signal transduction cascade initiated by PMA activation of PKC.

INTRODUCTION

Melanocytes are found distributed in several organs, such as the epidermis, the leptomeninges, the eye, and the inner ear (reviewed in Erickson, 1993). Embryologically, melanocytes share with sensory and sympathetic neurons a neural crest progenitor (reviewed in Holbrook *et al.*, 1988). This is demonstrated in a series of genetically transmitted human diseases like piebaldism and

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Waardenburg's syndrome that are characterized by defects in pigmentation (absence of melanocytes), deafness, and intestinal aganglionosis (Waardenburg's) (Lorton and Nordlund, 1991; Tassabehji *et al.*, 1992; Urabe *et al.*, 1993). When humans reach their fourth or fifth decade of life, the number of melanocytes in the epidermis, hair bulbs, eyes, and mucus membranes and the number of oral and cutaneous nevi begin to decrease (Nordlund, 1986). At the same time, there is a significant increase in the incidence of skin cancers. It is not known if the decrease in melanocyte numbers as humans age is because of premature senescence, terminal differentiation, or both phenomena. Possibly one result of the loss of melanocytes is a permissive environment for the development of malignancies.

The possibility to grow human melanocytes in vitro to study the cellular and molecular mechanisms involved in melanocyte migration, pigmentation, and differentiation has been achieved at the beginning of the last decade, using foreskin-derived cells from newborns, and growth promoters like phorbol 12-myristate 13-acetate (PMA), cholera toxin (CT), and isobutyl methylxanthine, and basic fibroblast growth factor (Eisinger and Marko, 1982; Gilchrest *et al.*, 1984; Halaban *et al.*, 1988; Herlyn *et al.*, 1988; Pittelkow and Shipley, 1989; Yaar and Gilchrest, 1991). In the last few years, new growth factors for melanocytes have been identified, such as mast cell growth factor (Funasaka *et al.*, 1992), hepatocyte growth factor (Matsumoto *et al.*, 1991), endothelins (Imokawa *et al.*, 1992), and the leukotrienes LTC_4 and LTD_4 (Morelli *et al.*, 1992).

Attempts to grow melanocytes derived from human adult epidermis in long term culture were unsuccessful because cells seemed unable to grow beyond the third passage in a growth medium containing CT and hypothalamic extracts (Gilchrest *et al.*, 1984). In the same medium melanocytes derived from newborns proliferated with a shorter generation time and for many more postprimary population doublings than human melanocytes. These differences in growth potential between cells from adults and newborns were attributed to the in vitro manifestation of aging. Human normal somatic cells from adults usually proliferate in vitro for ≥20–30 passages, depending on the donor's age. It is conceivable that the low passage number achieved by human adult melanocytes reflects an inherent short life span and/or that the growth factors selected for the growth of neonatal melanocytes are only transiently permissive for the growth of their adult counterparts. Recent data favor the last hypothesis, because in the presence of PMA or leukotriene C_4 (LTC_4), adult melanocytes proliferate for >30 cumulative population doublings (Medrano and Nordlund, 1990; Medrano *et al.*, 1993). We noted that just as in the skin, in the medium supplemented with PMA, these adult cells always maintained the territorial phenotype in which cell bodies never seem to have contact with each other. To decipher the discrepancies between the short proliferative life span observed in the medium supplemented with CT and the much longer one observed in medium supplemented with PMA, we analyzed the role of CT in normal human adult and neonatal melanocytes. We define here the culture conditions for terminal differentiation and characterize some of the cellular and molecular events associated with this phenotype. We observed that terminally differentiated melanocytes are epithelioid in shape with round, flat, large cell bodies. These morphological changes were preceded by a five- to sixfold increase in cAMP levels and an 8- to 10-fold increase in melanin content. Upon stimulation with PMA, senescent and terminally differentiated cells were unable to phosphorylate tyrosine residues of the mitogen-activated kinase extracellular signal-regulated kinase 2 (ERK2) (Boulton *et al.*, 1991). In addition, ERK2 did not show the nuclear accumulation observed in proliferating melanocytes after PMA stimulation. Our results suggest the existence of a maturation-dependent pathway of terminal differentiation and define a critical pathway similarly deficient in terminally differentiated and senescent cells.

MATERIALS AND METHODS

Skin Biopsy Specimens

Shave biopsies of ~1 cm^2 from normal human caucasian adults were obtained and processed as described (Medrano and Nordlund, 1990; Medrano *et al.*, 1993). The epidermal cell suspension obtained after trypsinization was seeded in medium GF, which consists of MCDB-153 (Sigma, St. Louis, MO), supplemented with crude fibroblast growth factor (0.3 ng/ml) (Collaborative Research, Waltham, MA), insulin (5 μg/ml), transferrin (5 μg/ml), α-tocopherol (1 μg/ml), 4% fetal bovine serum, catalase (20 μg/ml, added only during initial isolation) (all from Sigma), and pituitary extract (30 μg/ml) (Clonetics, San Diego, CA). When indicated, GF was supplemented with 8 nM PMA (GF/PMA) or 10 nM CT plus 0.1 mM 3-isobutyl-1-methylxanthine (GF/CT). Media were changed biweekly.

Cells

Melanocytes after 40–60 cumulative population doublings (CPD) in GF/PMA were considered senescent when they did not show any further increase in cell number during a 2-mo period. Likewise, melanocytes after 4–8 CPD in GF/CT were considered terminally differentiated when they did not show any increase in cell number during a 2-mo period.

cAMP Measurements

Cells growing in GF/PMA medium were shifted to CT for 4–6 d before cAMP analysis. Cellular cAMP content was determined with the ^{125}I cAMP kit from New England Nuclear (Boston, MA), according to the instructions of the manufacturer.

Melanin Assays

Melanin content was determined by absorption at 475 nm as described by Gordon and Gilchrest (1989).

Immunoblots

Total cell lysates were prepared by lysing the cells in RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, sodium salt (DOC), 0.1% sodium dodecyl sulfate [SDS], 50 mM tris(hydroxy-

methyl)aminomethane [Tris] pH 8.0) (Harlow and Lane, 1988) containing 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 100 μM sodium orthovanadate. After shearing and centrifugation, lysates were normalized for total cellular protein, and 150 μg of protein from each lysate was fractionated on a 7.5% SDS-polyacrylamide gel. Immunoblots were incubated overnight with a 1 μg/ml monoclonal phosphotyrosine antibody (PY-20, ICN, Costa Mesa, CA) in buffer A (5% Cohn crystallized bovine serum albumin [BSA] [ICN], 170 mM NaCl, 0.2% NP-40, 50 mM Tris pH 7.5). Filters were washed four times in buffer B (buffer A without BSA), incubated with buffer A for 30 min, and followed by a 1:15 000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG. Immunoreactivity was determined using the enhanced chemiluminescence (ECL) reaction (Amersham, Arlington Heights, IL). The blots were exposed to Kodak AR5 x-ray film (Rochester, NY). The same blot was stripped according to ECL protocols and probed with ERK1 (recognizes both ERK1 and ERK2, a gift from G.D. Yancopoulos, Regeneron Pharmaceuticals, and a rabbit polyclonal Ab [Ab 691] purchased from Santa Cruz Biotechnologies, Santa Cruz, CA). Blots were again stripped and probed with an ERK2 specific antibody (ERK2-NT, Upstate Biotechnology, Lake Placid, NY). Both antibodies were dissolved in buffer A containing 5% nonfat milk. Detection was as above using the ECL method.

In Gel Kinase Assay

Mitogen-activated protein kinase (MAPK) activity was analyzed according to the method of Kameshita and Fujisawa (1989) and Gotoh *et al.* (1991) with some modifications. Briefly, 15 μg of cell lysates were resolved on a 7.5% mini-acrylamide gel with 0.4 mg/ml of myelin basic protein copolymerized in the running gel. After electrophoresis, the gel was washed twice in 50 mM Tris pH 8.0 plus 20% 2-propanol. The gel was then re-equilibrated in buffer A (50 mM Tris pH 8.0, 5 mM mercaptoethanol) for 1 h, followed by two changes in 6 M guanidine HCl in buffer A, to denature proteins. Afterward proteins were renatured during a 72-h period in six washes in buffer A containing 0.04% Tween 40. We found that increasing the renaturation time from 16 to 72 h improved notably the activity of ERK2. The renatured proteins were incubated in MAPK buffer containing 25 mM Tris pH 7.2, 10 mM $MgCl_2$, 0.3 mM sodium orthovanadate, and 2 mM dithiotreitol at room temperature for 30 min. Phosphorylation reactions were initiated by layering 2.5 ml of buffer A containing 50 μM ATP and 100 μCi of [γ-^{32}P]ATP onto the gel and incubating at room temperature for 30 min. The reaction was stopped by removing the kinase solution followed by addition of a solution of 5% trichloroacetic acid containing 1% sodium pyrophosphate. After extensive washes in this solution during a 16-h period, gels were dried and autoradiographed by exposing them for 3 h to Kodak X-Omat film with an intensifying screen.

Protein Kinase C (PKC) and Protein Kinase A (PKA) Activity and Western Blot for PKC

The activity of both enzymes was determined as previously described using histone type III-S as a substrate for PKC and histone type II-AS for PKA, respectively (Park and Campisi, 1990). The cell lysates prepared for PKC activity were also used for Western blotting. Blots were probed with a PKC antibody from Seikagaku International (Rockville, MD).

Electron Microscopy

Melanocytes cultured in Tissue-Tek chamber slides were fixed and processed as described (Boissy *et al.*, 1991).

Immunocytochemistry

Cells, either proliferating or senescent, were seeded in Lab-Tek chambers precoated with fetal calf serum (FCS). After 3 d the growth medium was removed and replaced by MCDB-153 with no additions for 2 h. Control cells remained unstimulated, whereas the rest were stimulated with 100 nM PMA for 15 and 45 min. Afterward, chambers were washed two times with phosphate-buffered saline (PBS) and fixed with formalin for 15 min at room temperature. Cells were permeabilized with 0.5% Triton X-100 in PBS at room temperature followed by a 10-min treatment with 5 mM glycine in PBS. Blocking unspecific sites was performed with 5% normal goat serum in a solution containing 1% BSA in PBS. Cells were incubated with a 1/100 dilution of the ERK antibody 691 followed by a fluorescein-conjugated sheep anti-rabbit serum (Sigma) diluted in a solution containing 0.1% Triton X-100, 1% BSA in PBS.

RESULTS

PMA as a Melanocyte Mitogen: Proliferation to Senescence

Normal human cells in culture have a limited capacity to proliferate. This decline in capacity to proliferate has been termed the finite life span phenotype or cellular senescence (Hayflick, 1965). In medium MCDB-153 supplemented with PMA (GF/PMA), adult human melanocytes proliferate for >40 CPD (Table 1, lane 1). Melanocytes from neonatal foreskins also proliferate vigorously in this medium, reaching 60–90 CPD and higher saturation densities than adult cells (Table 1, lane 3). During the proliferative phase in GF/PMA, adult cells exhibit a bipolar-dendritic morphology and prominent nuclei (Figure 1A). At later passages (>30) these same cells became progressively more pigmented and spindle shaped (Figure 1B). Such cells are irreversibly growth arrested (senescent) and cannot be induced to divide with any human melanocyte mitogen. A characteristic feature of the senescent cultures is the presence of numerous multinucleated cells (Figure 1B inset).

CT as a Mitogenic-Melanogenic Agent: Proliferation to Terminal Differentiation

Agents that raise cAMP levels are mitogens for neonatal foreskin melanocytes (Eisinger and Marko, 1982; Gil-

Table 1. Influences of the growth medium on passage number and saturation density in melanocyte cultures derived from adult or neonatal skin

Melanocyte origin	Growth medium	Cumulative population doublings	Saturation density (cell # × 10^{-4}/cm^2)
Adult	GF/PMA	>40*	2–2.5
Adult	GF/CT	4–8	2–2.5
Neonatal	GF/PMA	>50 (60–90)	8–9
Neonatal	GF/CT	>20	2–3

Epidermal cell suspensions from adult human donors were prepared as described in MATERIALS AND METHODS. Each experiment represents the results and growth characteristics observed in cultures established from four or more donors.

* The number of population doublings (CPD) of melanocytes derived from adults was obtained using donors between 20 and 35 years of age. CPD decreased as a function of age for individuals >50 years of age.

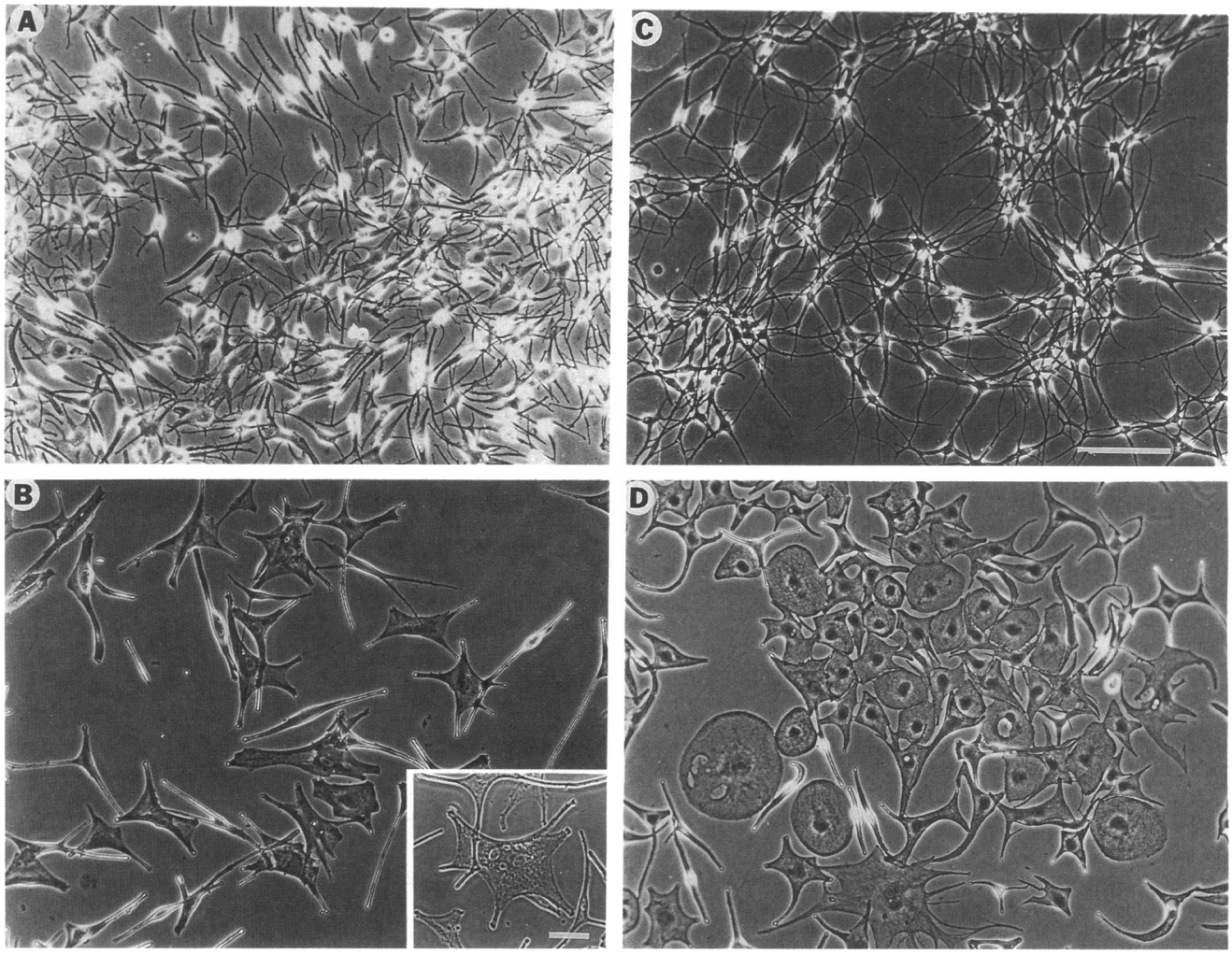

Figure 1. Proliferating, senescent, and terminally differentiated melanocyte phenotypes. (A) Proliferating, nonpigmented cells at an early passage in GF/PMA (4–6 CPD). (B) A senescent culture of A cells in the same GF/PMA medium at a late passage (60 CPD). Cells ceased dividing, became enlarged, and showed an increased number of multinucleated cells (an example of a multinucleated cell is shown in inset). (C) Cells from a culture initiated in GF/CT at an early passage (4 CPD). (D) Cells from C 1 mo later (8 CPD). Note the appearance of round, flat, and large cells. A–D pictures were taken at the same magnification. Bars, 100 μm.

chrest *et al.*, 1984; Halaban *et al.*, 1988; Herlyn *et al.*, 1988; Yaar and Gilchrest, 1991). Cultures from adult melanocytes initiated in medium MCDB-153 supplemented with CT (GF/CT) also showed an initial rapid proliferation. After 6–8 wk in this medium, adult cells became extremely pigmented. The morphology changed from dendritic in the early proliferative phase (Figure 1C) to epithelioid, round, or stellate cell (Figure 1D). These melanocytes were irreversibly arrested in the phases G_0G_1-G_2M of the cell cycle and could not be induced to divide by other melanocyte mitogens. Morphological changes and hyperpigmentation in melanocytes similar to those elicited by CT were previously observed in the human epidermis after sunlight exposure of the skin (Quevedo *et al.*, 1986) and in cultured neonatal melanocytes irradiated with UV light (Abdel-Malek, personal communication). During the early proliferative phase in GF/CT, adult cells can be switched to medium GF/PMA in which they proliferate with no appreciable loss of the proliferative potential. Once cells become quiescent in GF/CT, PMA was incapable to reinduce proliferation (Table 2). Cells that have ceased dividing and cannot be recalled into the cell cycle are by definition terminally differentiated cells (Baserga, 1985). The same phenomenon of CT-induced terminal differentiation was observed when adult melanocytes chronically growth stimulated by LTC_4 were switched to GF/CT, either in the presence or absence of LTC_4 (Medrano *et al.*, 1993). Cellular spheroids and individual cells became extremely pigmented and irreversibly growth arrested. On the contrary, the medium GF/CT allowed long term proliferation of neonatal melanocytes (Table 1, lane 4), although in this medium cells reached a lower saturation density than in GF/PMA. Others

Table 2. cAMP levels and melanin content in melanocytes derived from human adult epidermis

Growth conditions for adult-derived melanocytes	cAMP levels pmoles/10^6 cells	Melanin content pg/cell	Reversibility by PMA
GF/PMA	0, 6	5.5	—
GF/CT (proliferative phase)	4, 4	N.D.	yes
GF/CT (quiescent phase)	1.3	28.0	no
GF/CT/PMA	N.D.	5.5	no

Melanocytes at 10–14 CPD in GF/PMA medium were split and seeded in media containing either 8 nM PMA (GF/PMA), 10 nM CT plus 0.1 mM IBMX (GF/CT), or 8 nM PMA + 10 nM CT (GF/PMA + CT). cAMP assays for cells in the proliferative phase in GF/CT were performed 4 d after the shift to this medium. Melanin content was performed 20 d later. cAMP assays for quiescent cells were performed in cultures in GF/CT with no detectable proliferation capacity. To determine the reversibility of cells to PMA, proliferating or quiescent cultures in GF/CT were trypsinized, counted, and seeded in GF/PMA. Cell counts were determined 15 and 30 d later. Cells were considered to be unable to reverse to the proliferating phenotype when no appreciable increase in cell number was observed after 30 d in GF/PMA. N.D., nondetermined.

noted that neonatal melanocytes treated with 170 nM PMA, a dose more than 20 times higher than that used in our cultures, also displayed an epithelioid morphology and G_2M arrest (Chao-Hsing and Hsin-Su, 1991). The significance of these latter findings is not clear.

Ultrastructure of Proliferating, Senescent, and Terminally Differentiated Melanocytes

Electron microscopic analysis performed on cultured melanocytes showed that during the proliferative phase and after reaching senescence or terminal differentiation the cells always maintained the differentiated phenotype, i.e., the presence of melanin and melanosome organelles at different stages of maturation. However, there are qualitative and quantitative differences when senescent and terminally differentiated cells are compared to proliferating melanocytes (Figure 2, a–c); senescent and terminally differentiated cells have an increase in number of, and melanin deposition in, melanosomes (Figure 2, b and c). In addition, terminally differentiated cells contain melanosomes aggregated in structures tentatively identified as autophagosomes (Figure 2c). These structures sometimes occupied one-third to one-fourth of the cell. These results obtained in vitro correlate with observations made by other investigators using skin biopsies from elderly subjects (Breathnach *et al.*, 1991), whereby some melanocytes were congested with stage 3 and 4 melanosomes. It was previously suggested that when UV irradiation or other environmental injuries are repeated for prolonged periods, the cells undergo changes resembling those in aging cells (Breathnach *et al.*, 1991). Therefore, we speculate that CT mimics some of the effects of UV light observed in human epidermal melanocytes.

CT-associated Increase in cAMP and in Melanin

Replacement of GF/PMA with GF/CT causes the intracellular concentration of cAMP levels and melanin content to increase dramatically compared to cells left in GF/PMA (Table 2, lanes 1–3). As cells enter quiescence, cAMP levels were still higher than those obtained for proliferating cells in GF/PMA. It was observed previously that in skin biopsies obtained from normal individuals, addition of the cAMP analogue 8-bromo-

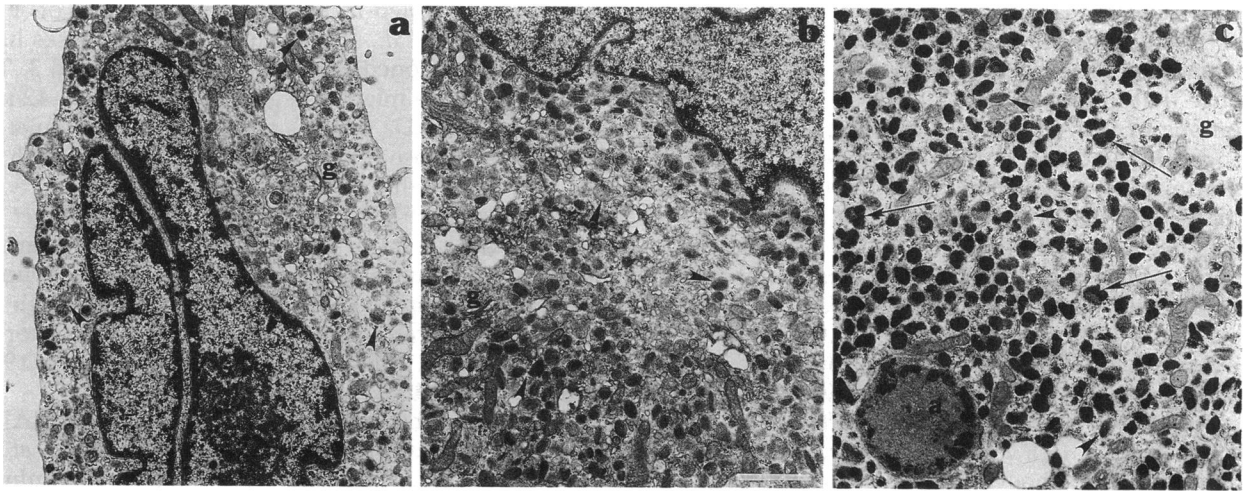

Figure 2. Ultrastructure of proliferating, senescent, and terminally differentiated melanocytes. (a) Proliferating cells in GF/PMA. (b) Senescent cells in the same medium as a. Most of the melanosomes are individually dispersed throughout the cytoplasm. (c) Terminally differentiated cells in GF/CT. Membrane bound compartments of melanosomes (arrows) were observed. These compartments ranged dramatically in size. Some were relatively small whereas others were extremely large and occupied as much as one-quarter of the cytoplasmic area. Putative autophagosomes (a) are also observed in terminally differentiated cells. g, Golgi apparatus. Bar, 2.0 μm.

cAMP to the culture medium increased the amount of tyrosinase (Burchill *et al.*, 1990). cAMP-related pigmentation and dendricity were also observed in neonatal foreskin-derived melanocytes grown in a CT supplemented media (Abdel-Malek *et al.*, 1992). Thus the morphological and biochemical changes observed in adult cells in GF/CT might be part of the signal transduction pathway associated with pigmentation and not the cause of terminal differentiation. When both PMA and CT were present (GF/PMA + CT), cells did not increase their melanin content (Table 2, lane 4; Figure 3); nevertheless, they became irreversibly growth arrested (Medrano and Nordlund, 1990). This latter observation shows that it is possible to uncouple pigmentation from terminal differentiation and that the effect of CT is dominant over the proliferative effect of PMA.

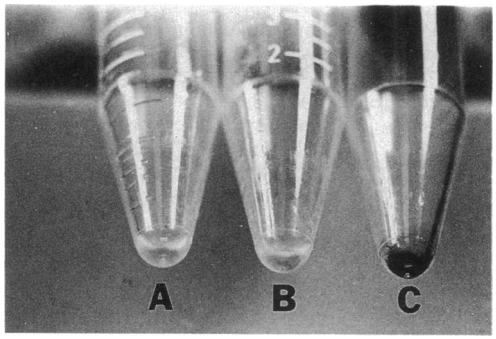

Figure 3. Uncoupling pigmentation from terminal differentiation. (A) Unpigmented cell pellet derived from a caucasian donor at 20 CPD in GF/PMA. (B) Same donor cell pellet, obtained 4 wk after the shift to GF/PMA + CT. (C) Same donor cell pellet, now exhibiting a very dark pigmentation, obtained 4 wk after the shift to GF/CT.

PKC and PKA in Proliferation and Quiescence

Phorbol esters (PMA) activate PKC and, after a chronic treatment, induce downregulation of the enzyme (Nishizuka, 1992). In turn, cAMP inducers like CT are known to activate PKA (Rozengurt *et al.*, 1988). To determine how these enzymes are affected by proliferation or terminal differentiation, we measured enzyme activities in adult and neonatal melanocytes under conditions of proliferation and terminal differentiation. As expected, proliferating cells in GF/PMA showed a much lower PKC activity than proliferating cells (2 CPD) in GF/CT (Figure 4, A and C). Western blot analysis confirmed the loss of PKC at the protein level rather than inactivation of the protein (Figure 4B). PKC is still downregulated by PMA in nonpigmented, terminally differentiated melanocytes in a medium containing both PMA and CT (GF/PMA + CT) (Figure 4A). These results and the one shown in Figure 3 support the hypothesis that PKC is involved in pigmentation (Park *et al.*, 1993), because cells with low PKC activity are also nonpigmented. This idea is supported further by the evidence that in neonatal melanocytes increasing concentrations of PMA in a medium supplemented with CT and IBMX induced a dose-dependent decrease in tyrosinase activity and a concomitant increase in cAMP (Abdel-Malek *et al.*, 1992).

It has been reported that growth of neonatal melanocytes, and possibly adult melanocytes, is dependent on initial activation of PKC followed by downregulation (Brooks *et al.*, 1991; Arita *et al.*, 1992). Our data show two examples in which PKC activity does not correlate with cellular proliferation. 1) PKC activity had similar values in proliferating and terminally differentiated cells in GF/CT (Figure 4C). 2) It is downregulated by PMA in proliferating melanocytes in GF/PMA and in quiescent, terminally differentiated melanocytes in GF/PMA + CT (Figure 4A). These results predict that in melanocytes PKC activation and downregulation are not sufficient for proliferation and that pathways after PKC may be also be required. PKA activity was similar for proliferating or quiescent cells either from adults or neonates in GF/CT medium (Figure 4D) and also for cells in GF/PMA (6500 cpm/μg protein). Although a transient increase in PKA activity may have occurred, we did not see it because PKA (and also PKC) was measured under steady state conditions: proliferation or quiescence.

Tyrosine Phosphorylation and ERK_1/ERK_2 Expression in Proliferating, Terminally Differentiated, and Senescent Melanocytes

We decided to analyze pathways downstream of PKC that are known to be also affected by phorbol esters like the MAPKs. These enzymes, also called ERKs, are now considered to be a major signal transducer pathway because they are rapidly activated in response to ligand binding to receptors that have tyrosine kinase activity, G protein coupled-receptors, or activation of PKC (Lange-Carter *et al.*, 1993). Three related kinases have been recently cloned and named ERK 1, 2, and 3. The protein products migrate in gels as bands of 40–42 kDa (ERK2), 42–44 kDa (ERK1), and 45 kDa (Boulton *et al.*, 1991). This kinase family has been correlated with induction of growth or differentiation in many cell types (Cobb *et al.*, 1991; Howe *et al.*, 1992; Thomas *et al.*, 1992). ERK2 seems to play an important role in melanocyte proliferation, because it is rapidly phosphorylated after treatment of neonatal melanocytes with the mitogens stem cell factor, hepatocyte growth factor, or basic fibroblast growth factor (Funasaka *et al.*, 1992; Halaban *et al.*, 1992).

Because phorbol esters are known to activate MAPKs in other cell types (Boulton *et al.*, 1991; Howe *et al.*, 1992; Thomas *et al.*, 1992), we wished to determine if ERK2 is activated by PMA, a major mitogen for melanocytes derived from human adults. For that we used proliferating melanocytes at early and late passage (CPD 12 and 40, respectively). Figure 5A shows the kinetics of MAPK activation by 100 nM PMA in early passage

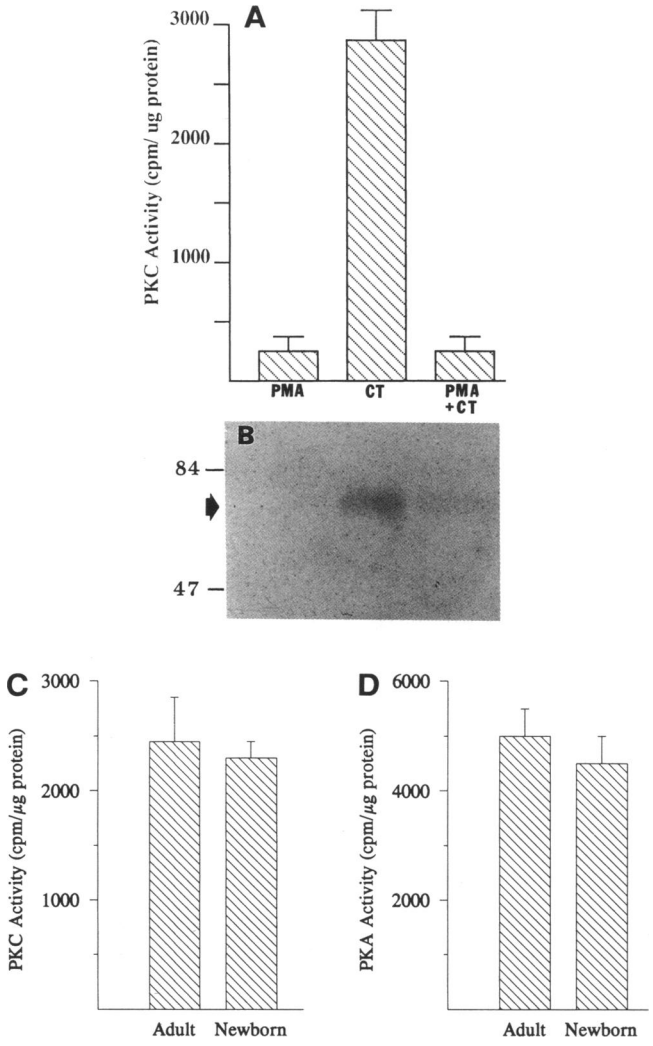

Figure 4. PKC activities in proliferating and terminally differentiated melanocytes from adult or neonatal origin. (A) PKC activity was determined in melanocyte cultures maintained in PMA (proliferating cells, GF/PMA), CT (terminally differentiated cells, GF/CT), or CT plus PMA (also terminally differentiated cells, GF/PMA + CT). (B) Western blot from cell extracts derived from A. Aliquots from experiment A were run in 8% SDS-polyacrylamide gel, blotted, and probed with a specific PKC Ab. The blot was developed using the ECL method. (C) PKC activity in adult and neonatal melanocyte cultures maintained in GF/CT. The enzymatic activity was assayed in cell extracts obtained from terminally differentiated cells (adults) and proliferating cells (neonatal). Bar, mean ± SE from three separate experiments. (D) PKA activities from adult and neonatal melanocyte cultures maintained in GF/CT. The enzymatic activity was assayed in cell extracts obtained from terminally differentiated cells (adults) and proliferating cells (neonatal). Bar, mean ± SE from three separate experiments.

cells, determined by immunoblotting with a polyclonal ERK1/ERK2 antibody (antibody 691). As previously reported for other melanocyte mitogens (Funasaka *et al.*, 1992; Halaban *et al.*, 1992), PMA activated ERK2 inducing a slow migrating form of the kinase. The addition of PMA seems to also increase ERK2 expression; further studies will be necessary to understand the mechanism of such modification. By overexposing the same blot, we also detected a set of two, apparently undescribed proteins, with molecular weights of 36 and 38 kDa, respectively (Figure 5B). These proteins seemed to follow the same temporal activation induced by PMA on ERK2. The biological consequences of these activations remains to be determined. To confirm that ERK2 is truly involved in PMA activation, blots were stripped, washed, and reprobed with a specific ERK2 monoclonal antibody. ERK2 showed a shift to slow migrating forms (Figure 5C), indicating possibly, posttranslational modifications by phosphorylation. Stripping and reprobing the same blot with a specific ERK1 monoclonal antibody did not show any shift to slow migrating species. Figure 5, D–F show that late passage melanocytes showed almost identical kinetics of ERK2 and p36-p38 activation.

Next, we wished to determine if ERK2 is phosphorylated in tyrosine and if it undergoes identical activation by PMA in senescent and terminally differentiated cells. Using an antiphosphotyrosine antibody, we observed that a treatment with 100 nM PMA for 15 min induced the following changes: 1) dephosphorylation of a protein of ~150 kDa, 2) increased phosphorylation of proteins in the 60–80 kDa range, and 3) a prominent phosphorylation of a 42 kDa protein (Figure 6A). We did not detect tyrosine phosphorylation in p36–p38, suggesting that either the total amount of protein loaded in the gel was not enough for their detection or that these two proteins are phosphorylated in amino acids other than tyrosine. After stripping and washing, the same membrane was probed with antibody 691. In unstimulated cells both proteins are readily detected, although ERK2 seemed to be more abundant than ERK1 (Figure 6B, lane 1). After PMA stimulation, both bands became less defined (Figure 6B, lane 2) and showed comigration with the 42 kDa phosphorylated band. To better discriminate ERK2 from ERK1, the membrane was stripped again, washed, and reprobed with a specific ERK2 antibody (ERK2-NT). We observed a clear shift in the mobility of ERK2, which comigrated with the phosphorylated band observed in cell extracts obtained after PMA stimulation (Figure 6C, lane 2). This observation suggests that the 42-kDa phosphorylated protein in fact is the kinase ERK2. The analysis of tyrosine-phosphorylated proteins in senescent and terminally differentiated cells revealed that ERK2 was no longer phosphorylated when PMA was added as a stimulus (Figure 6D, lanes 3 and 5), whereas it induced phosphorylation in ERK2 in early passage melanocytes that were still in their proliferative phase in GF/CT. The lack of phosphorylation in senescent and terminally differentiated cells occurred in spite of detectable levels of ERK2 protein. To determine whether a decline in ERK2 activity correlates with a decline in cell proliferation, we employed the technique of in gel kinase assay. We found that PMA strongly activated ERK2 in cells at low CPD and that the PKC inhibitor H-7 decreased such activity (Figure 6F, lanes 3 and 4). Slowly proliferating

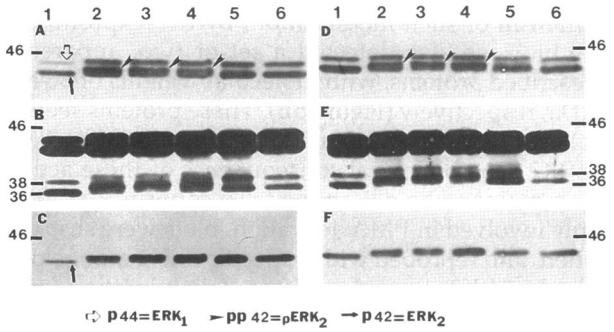

Figure 5. Time course of ERK2 activation by PMA in proliferating early and late passage melanocytes. Proliferating cells at 12 and 40 CPD in GF/PMA were seeded in p-100 dishes and maintained with no medium changes for 4 d. To downregulate growth signals cells were shifted for 2 h to the basal medium MCDB lacking serum and growth factors. Afterward the cells were stimulated with 100 nM PMA. The numbers 1–6 indicate: 0, 15 min, 30 min, 1 h, 3 h, and 5 h after PMA. Cell extracts were made in RIPA buffer containing sodium orthovanadate and a cocktail of protease inhibitors (see MATERIALS AND METHODS). Protein extracts (150 μg) were run in 7.5% SDS gels. A and D show blotted proteins from early and late passage cells probed with the MAPK antibody 691. B and E are the overexposed A and B blots to show p36 and p38 proteins. C and F are again blots A and D stripped as recommended by ECL protocols and reprobed with a specific ERK2 antibody (ERK2-NT).

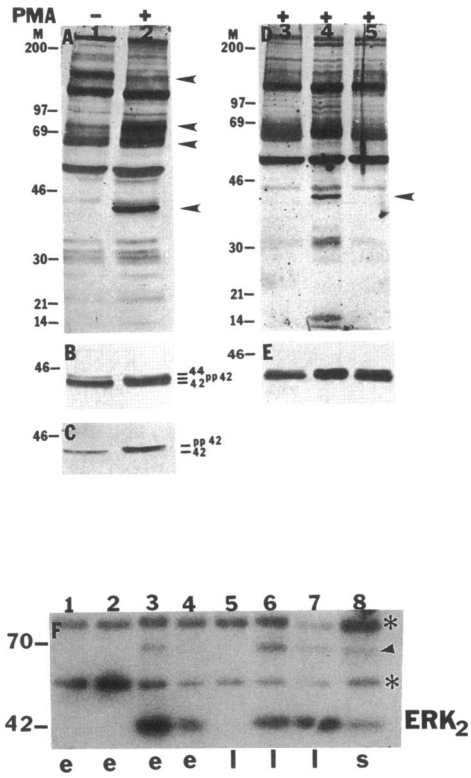

Figure 6. PMA-induced tyrosine phosphorylation and MAPK activation in human adult melanocytes. Repression of ERK2 phosphorylation in senescent and terminally differentiated cells. (A) Tyrosine phosphorylation in proliferating melanocytes cultured in GF/PMA. Melanocytes at an early passage (10–14 CPD) were seeded in p-100 dishes and maintained with no medium changes for 4 d. To downregulate growth signals cells were shifted for 2 h to the basal medium MCDB lacking serum and any other growth factors. Afterward the cells were left in the same medium (lane 1) or stimulated with 100 nM PMA for 15 min (lane 2). After blotting proteins to nitrocellulose, membranes were probed with 1/500 dilution of an anti-phosphotyrosine antibody (P-Y 20, ICN). Arrows indicate, from the top to the bottom of the gel, the dephosphorylation of a 150-kDa protein and the increase in phosphorylation in proteins of 60–80 and 42 kDa, in PMA-treated cells. (B) The same blot was stripped, washed, and probed with antibody 691. (C) Again the same blot was stripped, washed, and probed with a specific ERK2 antibody (ERK2-NT, UBI). (D) Phosphotyrosine proteins in senescent and terminally differentiated melanocytes. Senescent melanocytes after 60 CPD in GF/PMA (lane 3), proliferating melanocytes after 2–4 CPD in GF/CT (lane 4) and terminally differentiated melanocytes after 8 CPD in GF/CT (lane 5) were seeded in p-100 dishes and left in the same medium for 4 d. All cultures were shifted for 2 h to the basal medium MCDB as described above. Afterward, cultures were stimulated with 100 nM PMA for 15 min. Only proliferating cells were able to show phosphorylation in a 42-kDa protein. (E) The same blot was washed and reprobed with Ab 691. (F) In gel kinase assay in melanocyte extracts at 10 CPD (e, early passage, actively proliferative phase), at 50 CPD (l, late passage cells with diminished proliferative rates), and at 60 CPD (s, senescent cells), was performed as described in MATERIALS AND METHODS. Cell extracts were prepared from cells either untreated (lanes 1 and 5) or treated with 100 nM PMA for 15 min (lanes 3, 6, and 8). Cell extracts were prepared from cells that had been pretreated for 60 min with 50 μM H-7, before the addition of PMA (lane 2), or pretreated with H-7 and incubated with PMA (lanes 4 and 7, respectively). Bands marked by an asterisk possibly represent autophosphorylation by the renatured kinases. The band marked by an arrowhead represents an unidentified kinase that is apparently PMA dependent.

cells at a late passage (50 CPD) showed a diminished ERK2 activity and H-7 was less effective as PKC inhibitor (Figure 6F, lanes 6 and 7). An almost complete loss in ERK2 activity is dramatically observed in senescent cells (Figure 6F, lane 8). Interestingly, an unidentified 70-kDa kinase, apparently PMA-dependent (Figure 6F, arrowhead, lanes 3, 6, and 8), showed little or no dependence with the proliferative status of the cells.

Immunolocalization of ERKs

It has been shown in COS-7 cells transiently transfected to express ERK2 (Seth *et al.*, 1992) that ERK2 accumulates in the nucleus upon serum stimulation. This phenomenon is also observed in the chicken hepatoma DU249 cells (Sanghera *et al.*, 1992) and in HeLa cells (Chen *et al.*, 1992). Therefore, we investigated the cellular distribution of ERK1/ERK2 in normal proliferating and senescent melanocytes. Unstimulated, proliferating melanocytes showed a mostly perinuclear punctate, diffuse distribution (Figure 7A). As a result of PMA stimulation there was a marked increase in nuclear staining clearly observed 15 and 45 min after PMA treatment (Figure 7, B and C). Large, senescent melanocytes, stimulated with PMA, showed ERK2 accumulation in the vicinity of the nuclear envelope (Figure 8B, arrowheads) and no appreciable ERK2 accumulation in the nucleus (Figure 8, A–C). Partial accumulation in the nucleus was observed only in some bipolar cells of the same culture. To avoid any misinterpretation of these results because of differences in cell size between proliferating and senescent cells, we used the melanoma cell line

UCD-Mel-N as a positive control. These melanoma cells displayed a flatter and larger cytoplasm compared to normal proliferating melanocytes, allowing ERKs to be clearly observed in the cytoplasm outside the nucleus and their accumulation in the nucleus 15 min after PMA stimulation (Figure 8, D and E). The UCD-Mel-N cells also showed tyrosine phosphorylation in ERK2 and p36 activation after PMA stimulation (Figure 8, F–H) with kinetics identical to proliferating melanocytes (Figure 5 and 6).

DISCUSSION

Melanocytes are one of the three major populations of cells that compose the human epidermis. One important function of melanocytes is to produce melanin after exposure to inflammatory stimuli of all types. In recent years, important progress has been made regarding the biochemical and molecular mechanisms of pigmentary disorders (reviewed in Hearing, 1993), but studies about the life cycle of the melanocyte either in vivo or in vitro are still in its infancy. Recently it has been found that the β isoform of PKC may regulate human melanogenesis by activation of tyrosinase (Park *et al.*, 1993). Our results indicate that in the melanocyte there are at least two events associated with pigmentation and manifested when cells are in GF/CT, i.e., increase in cAMP and in melanin content. In parallel, the levels of PKC are three to four times higher than the one found for cells grown in the presence of PMA. Thus, we predict that in cells treated with CT, the activation of cAMP- and PKC-dependent pathways would result in the increase of the end product, melanin. Similar observations have been made previously for neonatal melanocytes growing in a medium supplemented with PMA, CT, and 3-isobutyl-1-methylxanthine (IBMX) (Abdel-Malek *et al.*, 1992). We observed that melanocytes also increased their melanin content as they aged in vitro. This increase in melanin in aged cells is less dramatic than the increase observed in terminally differentiated cells and could be explained by the reduced activity of tyrosinase observed in cells treated with phorbol esters compared to other growth promoters like LTC_4 (Medrano *et al.*, 1993).

Adult and neonatal-derived melanocytes differed in the proliferative capacity stimulated by CT, because the adult-derived cells showed a gradual commitment to terminal differentiation at a much earlier time period than their neonatal counterparts. The ability of cells reverting toward a proliferative phenotype in a PMA containing medium suggests a stochastic commitment to terminal differentiation (Bennett, 1989). Undoubtedly, CT may not be the only factor able to induce terminal differentiation in the melanocyte. We observed that rising Ca^{2+} levels to 1.5–2.0 mM shortened the differentiation program initiated by CT. Other candidates may include compounds and agents that are known to modulate cAMP-PKA pathways including members of the melanotropin family, prostaglandins (Abdel-Malek *et al.*, 1988), and ultraviolet radiation (UVR) (Quevedo *et al.*, 1986). Based on the fact that activation of the MAPK pathway seems to be an obligatory pathway in the response elicited by many growth factors, we chose to analyze PMA-dependent phosphorylation events and to compare them with the pattern obtained under same

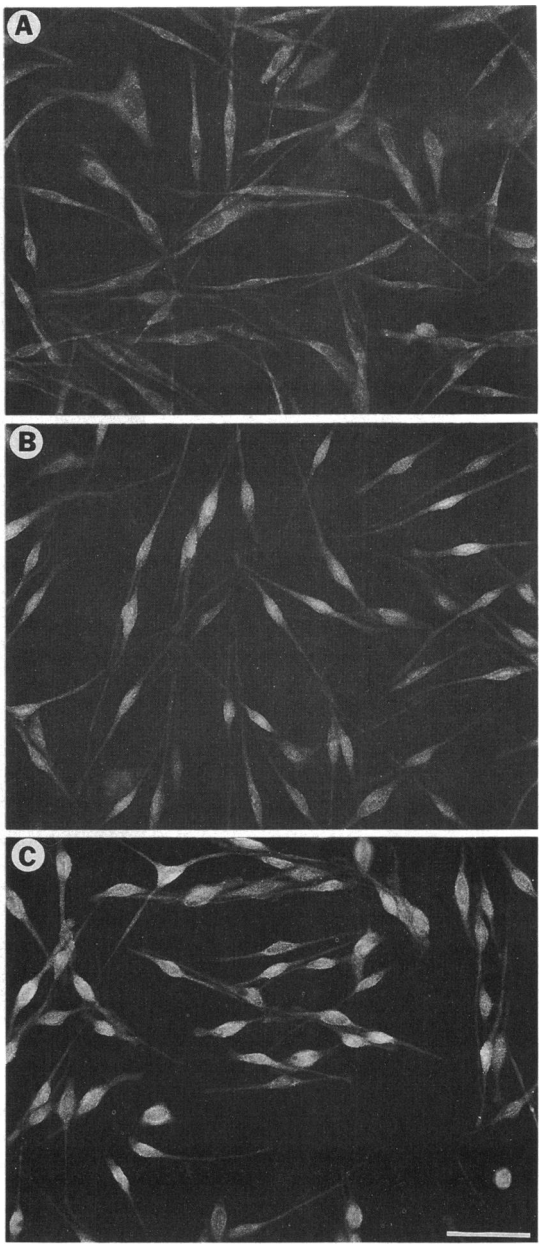

Figure 7. Immunocytochemical localization or ERKs in proliferating melanocytes. Melanocytes in GF/PMA were seeded in Lab-Tech chambers precoated with FCS. After 3 d, growth signals were down-regulated as for Figures 5 and 6. Afterward, cells were left unstimulated (A) or stimulated with 100 nM PMA for 15 (B) and 45 (C) min. Cells were fixed for immunocytochemistry as described in MATERIALS AND METHODS. Bar, 50 μ.

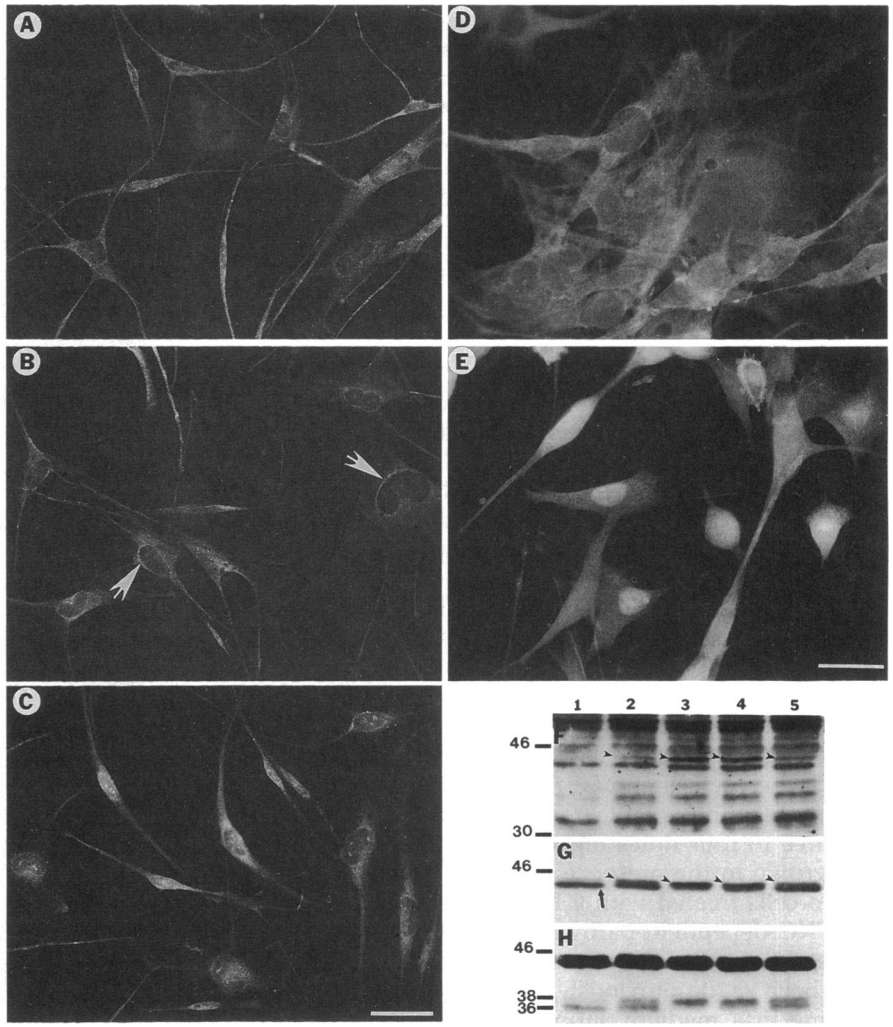

Figure 8. Immunocytochemical localization of ERKs in senescent melanocytes. Cells at 60 CPD were seeded and treated with PMA as for Figure 7. The human melanoma cells UCD-Mel-N were used as positive controls. (A) and (D) unstimulated melanocytes and melanoma cells, respectively. (B) and (E) melanocytes and melanoma cells 15 min after PMA stimulation. (C) melanocytes, 45 min after PMA stimulation. Bar, 50 μ. (F–H) Time course of PMA-induced events in the UCD-Mel-N cells. The numbers 1–5 indicate 0 min, 5 min, 15 min, 30 min, and 1 h after PMA. (F) Tyrosine phosphorylation in a 42-kDa protein. (G) The same blot probed with antibody 691. (H) Overexposure of G to show p36 activation by PMA.

stimuli in terminally differentiated and senescent cells. Both quiescent phenotypes failed to phosphorylate the ERK2 gene product and possibly a set of 32- and 14-kDa proteins. Moreover, ERK2 activity paralleled proliferation at early passages, decreased its activity when cells increased their generation times at late passages and showed minimal activity at senescence. PKC and a 70-kDa kinase was not obviously affected by the proliferation status of the melanocyte. In addition, no appreciable nuclear accumulation of ERK2 after PMA stimulation was observed. These events seem to correlate with the inability of terminally differentiated and senescent melanocytes to respond to the growth promoting signals induced by PMA. Possibly, at the onset of proliferation both neonatal and adult melanocytes share identical pathways. But progressively with time, there is an irreversible commitment to terminal differentiation of adult cells whereas neonatal cells sustain their proliferative capacity. Interestingly, neonatal melanocytes are less restricted to growth inhibition by high cell densities than adult melanocytes, a fact possibly associated with the expression of a still immature phenotype. One feasible explanation for the role of CT in terminal differentiation would be the induction of partial features of senescence early in the melanocyte lifespan. This hypothesis is based on morphological similarities between senescent and terminally differentiated cells but also on the identical, restricted pattern of tyrosine phosphorylated proteins and the inability to translocate to the nucleus when stimulated with PMA.

Cellular senescence has been studied most extensively in cultures of human fibroblasts (Hayflick, 1965). In these cells, senescence entails a partial reprogramming of the pattern of gene expression (Campisi, 1991). Cells fail to phosphorylate the retinoblastoma gene product (Stein *et al.*, 1990), to express cdc2, cycA, and cycB (Stein *et al.*, 1991) in response to mitogen stimulation, and to express the c-fos gene product (Seshadri and Campisi, 1990). Although these represent only a subset of mitogen-induced prereplicative events deficient in senescent cells, the vast majority of them are still functional (Stein *et al.*, 1991). Phosphorylation of Rb begins at the

onset of G_1 (Lin and Wang, 1992), and cdc-2 transcripts are high in S and G_2 phases, whereas fos requirements for entry into the S phase occur in early to mid G_1 (Muller *et al.*, 1984). In this sequence of events, the activation by phosphorylation of the MAPKs is now considered to precede c-fos activation (Gille *et al.*, 1992) because phosphorylation of the transcription factor $p62^{TCF}$ by MAPKs (ERKs) stimulates ternary complex formation at the c-fos promoter. These pathways link the expression of the human c-fos proto-oncogene to signal transduction pathways known to be activated before its own induction. The essential role the MAPKs have in cell cycle regulation is stressed further by the fact that inhibition of MAPK activation by the use of MAPK antisense RNA or by the expression of a MAPK dominant negative mutant, prevents G_0-arrested fibroblasts from entering the cell cycle (Pagès *et al.*, 1993). Based on these results, ERK2's inability to translocate to the nucleus could be considered a master stop sign that prevents the continuation of the signal transduction cascade initiated by PMA. However, as cellular senescence begins to be unraveled it is becoming increasingly clear that cells have multiple and possibly redundant stop signals (Rose *et al.*, 1992). In the same manner that no single target of the protein-tyrosine kinases is sufficient to mediate cell growth or transformation in normal human diploid fibroblasts (Cantley *et al.*, 1991), lack of ERK2 activation could be but one of the events that determine the end of the proliferative life span for the melanocyte. Because ERK2 protein is present at similar levels in either proliferating or senescent melanocytes, it is imperative to analyze the activity of the PMA-dependent activator of ERK2, MAP kinase (or ERK) kinase (MEK) (Lange-Carter *et al.*, 1993). In turn, MEK is known to be a convergence point for two different kinase pathways, Raf-1 and MAP kinase kinase (MEKK) (Lange-Carter *et al.*, 1993; Moodie *et al.*, 1993). Neither of them has been defined for the melanocyte. On the other hand, protein phosphatases, like the recently described MKP-1 that physically associates with MAPK (Sun *et al.*, 1993), could, by increasing their activities, be responsible for the loss of ERK2 activation observed in senescent and terminally differentiated melanocytes. Such studies are now in progress.

ACKNOWLEDGMENTS

We are grateful to Zalfa Abdel-Malek for her advice in the use of the cAMP kit and for her critical reading of the manuscript, to David Alcorta for supplying us with the protocol for the in-gel kinase assay, and to Ms. Ying Boissy for obtaining the electron micrographs. We also thank Joan Griggs for her help in editing the manuscript. This work was supported in part by the National Vitiligo Foundation, Tyler, TX; The Robert Gould Foundation, Cincinnati, OH; and grants from The American Federation for Aging Research, New York, NY; and 1K04 AGOO594-01 from the National Institutes of Health (to E.E.M.).

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