In B16 melanoma cells, mitogen-activated protein (MAP) kinases are activated during cAMP-induced melanogenesis (Englaro, W., Rezzonico, R., Durand-Clément, M., Lallemant, D., Ortonne, J. P., and Ballotti, R. (1995) J. Biol. Chem. 270, 24315–24320). To establish the role of the MAP kinases in melanogenesis, we studied the effects of a specific MAP kinase kinase (MEK) inhibitor PD 98059 on different melanogenic parameters. We showed that PD 98059 inhibits the activation of MAP kinase extracellular signal-regulated kinase 1 by cAMP, but does not impair the effects of cAMP either on the morphological differentiation, characterized by an increase in dendrite outgrowth, or on the up-regulation of tyrosinase that is the key enzyme in melanogenesis. On the contrary, PD 98059 promotes by itself cell dendricity and increases the tyrosinase amount and activity. Moreover, down-regulation of the MAP kinase pathway by PD 98059, or with dominant negative mutants of p21 ras and MEK, triggers a stimulation of the tyrosinase promoter activity and enhances the effect of cAMP on this parameter. Conversely, activation of the MAP kinase pathway, using constitutive active mutants of p21 ras and MEK, leads to an inhibition of basal and cAMP-induced tyrosinase gene transcription. These results demonstrate that the MAP kinase pathway activation is not required for cAMP-induced melanogenesis. Furthermore, the inhibition of this pathway induces B16 melanoma cell differentiation, while a sustained activation impairs the melanogenic effect of cAMP-elevating agents.

Melanocytes are specialized cells located at the basal layer of the epidermis that synthesize and transfer melanin pigments to surrounding keratinocytes leading thereby to a uniform skin pigmentation. In vivo, melanin pigments play a key photoprotective role against the carcinogenic effects of solar ultraviolet light, which is in other respects the physiologic stimulus of melanogenesis (1, 2). UV radiation can act directly on melanocytes or indirectly through the release of keratinocyte-derived factors that regulate melanogenesis (3, 4). Among the agents secreted by keratinocytes upon UV-B treatment, α-melanocyte-stimulating hormone (α-MSH) is one of the most potent activators of melanogenesis. Indeed, addition of α-MSH in cultured human melanocytes (5) or in melanoma cells (6) stimulates melanization. Further, subcutaneous injection of this hormone causes a strong stimulation of the local pigmentation in humans (7). α-MSH binds to a G protein-coupled heptahelical receptor leading to the activation of Gαq protein and to an increase in intracellular cAMP content. In cultured melanoma cells, the melanogenic effect of α-MSH can be mimicked by other cAMP-elevating agents such as cholera toxin, forskolin, and isobutylmethylxanthine (8–10). These observations emphasize the pivotal role of cAMP in the regulation of melanogenesis, but the cellular signaling events connecting the rise in cAMP to the stimulation of melanin synthesis are still incompletely clarified.

Melanin biosynthesis or melanogenesis consists in a cascade of enzymatic and spontaneous reactions that converts tyrosine to melanin pigments. The initial and rate-limiting step in melanin synthesis, the hydroxylation of tyrosine to DOPA, is controlled by tyrosinase that is the key enzyme in this process. Stimulation of melanogenesis by cAMP-elevating agents, as well as by other melanogenic agents, implies an increase in tyrosinase protein amount as the consequence of the stimulation of the tyrosinase gene transcription (11). Concerning the early events induced by cAMP increase, we have recently demonstrated that cAMP-elevating agents inhibit the phosphatidylinositol 3-kinase and p70S6 kinase activities (12). Further, the inhibition of these activities by pharmacological inhibitors mimics the melanogenic effect of α-MSH or forskolin, suggesting that phosphatidylinositol 3-kinase and p70S6 kinase inhibition by cAMP-elevating agents is a key event in the regulation of melanogenesis by these agents.

The MAP kinases ERK1 and ERK2 are serine/threonine kinases that are activated upon phosphorylation by the dual specificity MAP kinase kinase or MEK. MEK is phosphorylated and activated by Raf-1, which is itself activated by p21 ras (13). Upon activation, MAP kinases translocate to the nucleus where they phosphorylate and activate many transcription factors of which ternary complex factor/serum response factor and activator protein-1 are the most studied (14, 15). Although MAP kinases have been clearly shown to play a crucial role in growth control (16), they are also involved in the differentiation process of several cell systems (17, 18).

In a previous report, we have shown the activation of ERK1 during cAMP-induced melanogenesis in B16 melanoma cells (10). We have also observed a translocation of ERK1 to the nucleus, with a concomitant stimulation of activator protein-1 DNA binding activity. Further, stimulation of melanogenesis,
which is associated with morphological changes characterized by an increased cell dendricity, reflects the differentiation of melanocytes and melanoma cells. These observations led us to hypothesize that the MAP kinase pathway could be involved in the regulation of melanoma cell differentiation and, more precisely, that the activation of ERKs would be a required event in the induction of melanogenesis by cAMP-elevating agents in B16 melanoma cells.

In the present report, using a pharmacological approach with a specific inhibitor of MEK (PD 98059) (19) and a molecular approach with constitutively active or dominant negative mutants of MEK and p21<sup>ras</sup>, we clearly demonstrated that cAMP-elevating agents can stimulate melanogenesis in the absence of MAP kinase activation, thereby invalidating our former hypothesis. Further, we showed that a sustained activation of the Ras/MAP kinase pathway led to a down-regulation of melanogenesis, demonstrating the involvement of this pathway in the control of B16 melanoma cell differentiation.

EXPERIMENTAL PROCEDURES

Materials—Forskolin, TPA, 1-DOPA, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), aprotinin, and leupeptin were purchased from Sigma. Dulbecco's modified Eagle's medium, LipofectAMINE reagent, and OptiMEM medium were from Life Technologies, Inc. PD 98059 was from Santa Cruz Biotechnology. Polyclonal rabbit antisera to human tyrosinase (PEP-7) was provided by Dr. V. Hearing (Bethesda, MD). Antiserum to ERK1 was a generous gift from Dr. E. Van Obberghen (Nice, France). Expression vectors coding for the dominant positive and negative mutants of p21<sup>ras</sup>, respectively Val-12 p21 and Asn-17 p21 (20, 21), were provided by Dr. J.C. Chambard (Center de biochimie, Nice, France). Expression vector coding for constitutively active MEK (S222D) and constitutively inactive MEK (S222A) mutants were previously described (22).

Cell Cultures—B16/F10 murine melanoma cells were cultured in Dulbecco's modified Eagle's medium with 7% fetal calf serum (HyClone Laboratories) and penicillin/streptomycin (100 IU/50 μg/ml) in a humidified atmosphere containing 5% CO<sub>2</sub> in air at 37 °C.

Kinase Assay—Serum-starved cells were treated as indicated, rinsed, and solubilized in ice-cold lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10 mM Na<sub>4</sub>PO<sub>4</sub>, 2 mM NaF, 1% Triton X-100, supplemented with protease inhibitors, aprotinin, and leupeptin (2 μg/ml), leupeptin (10 μM), and AEBSF (1 mM)). Then extracts were clarified by centrifugation and incubated for 2 h at 4 °C with ERK1 antibody preadsorbed to protein A-Sepharose. Immune complexes were washed twice with lysis buffer and twice with HNTG buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, and 0.2 mM Na<sub>4</sub>VO<sub>4</sub>). Pellets were resuspended in 50 μl of HNTG buffer supplemented with 30 mM magnesium acetate, 15 μM cold ATP, and 0.2 mg/ml of mayer's protein (MBP) final concentrations. Reactions were initiated at room temperature by the addition of 3 μCi of [γ<sup>32</sup>P]ATP (3000 Ci/mmol) and stopped after 45 min by adding Laemmli sample buffer. Samples were then boiled for 5 min, and proteins were separated by SDS-polyacrylamide gel electrophoresis in a 12.5% acrylamide gel. The incorporation of <sup>32</sup>P was visualized by autoradiography. Then films were scanned and bands were quantitated by densitometry using MacBas software.

Determination of Tyrosinase Activity—Tyrosinase activity was estimated by measuring the rate of oxidation of L-DOPA (23). Cells grown in 6-well dishes were treated as indicated for 48 h in Dulbecco's modified Eagle's medium, 2% fetal calf serum. Then cells were washed in ice-cold phosphate-buffered saline and lysed in 100 μl of phosphate buffer (0.1 M, pH 6.8, containing 1% Triton X-100, 2 μg/ml apro- townin, 10 μg leupeptin, and 1 mM AEBSF). Cellular extracts were clarified by centrifugation at 13,000 × g for 5 min. The tyrosinase substrate 1-DOPA (2 mg/ml) was prepared in the same lysis phosphate buffer (without Triton). 40 μl of each extract were put in a 96-well plate, and the enzymatic assay was started by adding 100 μl of 1-DOPA solution at 37 °C. Control wells contained 40 μl of lysis buffer. Absorbance at 570 nm was read every 10 min for at least 1 h at 37 °C using a microplate reader (Dynatech Laboratories). The blank was removed from each absorbance value, and a plot of absorbance against time was represented for each condition. The final activity was expressed in ΔOD/min/μg of protein for each condition.

Western Blot Analysis—Cellular extracts were prepared as described above, and an equal amount of protein was separated by SDS-polyacryl-
B16 cells, grown in 2% FCS-containing medium (BASAL), were incubated for 48 h with 20 μM forskolin (FK) and/or with 10 μM PD 98059. Cells were photographed under phase-contrast microscopy. Bar represents 40 μm.

PD 98059 induces dendrite outgrowth and potentiates the morphological changes induced by cAMP-elevating agents.

**Inhibition of MAP Kinases by PD98059**

**Induces Melanogenesis**—Induction of melanogenesis in B16 melanoma cells is characterized by the stimulation of tyrosinase activity resulting from an increase in the tyrosinase protein expression. We therefore studied the effect of PD 98059 on these parameters. First, we measured tyrosinase activity in response to PD 98059. Cells were treated for 48 h with the MEK inhibitor and with forskolin. Then measured, in a cell-free system, the DOPA-oxidase activity that is the second specific activity of tyrosinase. As shown in Fig. 3A, forskolin stimulated about 8-fold the tyrosinase activity, and we observed a 6-fold stimulation with PD 98059. Addition of forskolin together with PD 98059 allowed us to achieve almost a 10-fold stimulation of tyrosinase activity, suggesting that inhibition of MAP kinases potentiates the effect of forskolin on tyrosinase activity.

We then measured the amount of tyrosinase protein in cells exposed to PD 98059. Cells were treated as described above, and Western blot analysis was performed using a specific antibody to tyrosinase (Fig. 3B). Forskolin or PD 98059 markedly increased the amount of tyrosinase protein compared with the weak level of expression in untreated cells. Concomitant treatment with forskolin plus PD 98059 resulted in an even more pronounced up-regulation of tyrosinase expression. Thus, inhibition of MAP kinases by PD 98059 is sufficient to stimulate tyrosinase expression and activity.

**Inhibition of MAP Kinases by PD98059 Stimulates the Tyrosinase Promoter Activity**—In a previous report we have shown that the effect of cAMP on melanogenesis is meditated through a stimulation of tyrosinase promoter activity, reflecting an activation of the tyrosinase gene transcription (11). Using a plasmid containing a 2.2-kilobase pair fragment of the tyrosinase promoter cloned upstream of the luciferase coding sequence as a reporter gene, we investigated whether the inhibition of MAP kinase led also to a stimulation of tyrosinase gene expression (Fig. 4). After transfection with the reporter plasmid, cells were incubated with forskolin, PD 98059, or forskolin plus PD 98059 for 48 h. We observed that PD 98059 alone stimulated luciferase activity 4-fold above the basal level. When forskolin was added to PD 98059, the luciferase activity reached a 9–10-fold stimulation, while forskolin alone induced a 8-fold stimulation. Hence, inhibition of the MAP kinase pathway by PD 98059 triggered an increase in tyrosinase gene expression and potentiate the effect of forskolin. It should be noted that the maximal effect on cell dendricity, melanogenesis, and tyrosinase promoter activity was observed with 10 μM PD 98059.

**Expression of Dominant Negative Mutants of MEK and p21ras Increases Basal and cAMP-induced Tyrosinase Promoter Activities**—To confirm the results obtained with the pharmacological inhibitor of MEK we have investigated the effect of dominant negative mutants of MEK and p21ras on the tyrosinase gene expression. p21ras is a small GTPase that activates Raf-1, the kinase that function upstream of MEK. These two mutants, that have respectively the ability to repress the endogenous activity of MEK and Ras, were cotransfected with the reporter plasmid (Fig. 5). In cells expressing the dominant negative mutants of MEK (MEK−) both basal and forskolin-stimulated tyrosinase promoter activities were increased about 3-fold compared with control conditions. The dominant negative mutant of Ras (Ras−) induced a 2-fold augmentation of the tyrosinase gene transcription in basal and forskolin-treated cells. Thus, inhibition of the Ras/MAP kinase pathway by dominant negative mutants of Ras and MEK stimulates basal and cAMP-induced tyrosinase promoter activities.

**Expression of Dominant Positive Mutants of MEK and Ras Inhibits Basal and cAMP-induced Tyrosinase Promoter Activities**—Since the inhibition of MAP kinases induces an augmentation of the tyrosinase gene expression, we wondered whether the expression of dominant positive mutants of p21ras (Ras+) and MEK (MEK+) would lead to an inhibition the tyrosinase gene promoter activity (Fig. 6). In cells co-expressing MEK+ and the reporter plasmid, we observed about a 2-fold decrease in luciferase activity in both basal and forskolin conditions. Expression of the dominant positive mutant of Ras (Ras+) appeared to be more potent to inhibit the forskolin-induced tyrosinase promoter activity. Thus, constitutively activation of the Ras/MAP kinase pathway leads to an inhibition of basal and cAMP-induced tyrosinase gene transcription.

**DISCUSSION**

The different approaches used in this report clearly demonstrate that the inhibition of the MAP kinase pathway by a pharmacological inhibitor of MEK or by dominant negative mutants of Ras and MEK does not prevent the effects of forskolin on different parameters reflecting the stimulation of melanogenesis. On the contrary, inhibition of the MAP kinase pathway is sufficient to induce dendrite outgrowth and to increase tyrosinase expression and activity. Further, sustained activation of this pathway, by over expression of constitutively active Ras and MEK, inhibits basal and cAMP-induced tyrosinase promoter activities. These findings invalidated our initial hypothesis suggesting that the activation of ERK1 by forskolin in B16 melanoma cells was a key event in cAMP-induced melanogenesis. However, our present results disclose the link between the MAP kinase pathway and the regulation of melanoma cell differentiation and demonstrate that the activation of the MAP kinase pathway leads to the inhibition of melanogenesis and differentiation.

At variance with our results, previous works have demonstrated that MAP kinases activation is required for the megakaryocytic differentiation of K562 cells (24, 25), the neuronal differentiation of PC12 (17) and the adipogenic differentiation of 3T3-L1 cells (18). However, concerning the rat pheo-
The inhibition of cell growth by serum depletion and the increase of melanogenesis, by the inhibition of MAP kinases, cannot be solely explained by an inhibition of cell growth. Indeed, UV-B irradiation that is a potent melanogenic stimulus inhibits growth of cultured human melanocytes (32). Growth factors such as basic fibroblast growth factor or TPA inhibit melanogenesis and stimulate growth of melanocytes (33). However, it should be noted that the stimulation of melanogenesis, by the inhibition of MAP kinases, cannot be solely explained by an inhibition of cell growth. Indeed, 10 μM PD98059 has only a very moderate inhibitory effect on growth of B16 cells, while inhibition of cell growth by serum depletion leads to faint increase in melanogenesis.

Additionally, it should be noted that down-regulation of the
MAP kinase pathway is thought to trigger cell cycle arrest in the G1 phase (16). During this phase, a number of cyclin-dependent kinases including Cdk2, 4, 5, and 6 are accumulated (34). Interestingly, it has been recently shown that Cdk5 and its associated regulator protein p39 plays a critical role in neurite outgrowth during neuronal differentiation (35). We can therefore envision that the inhibition of the MAP kinase pathway would result in the accumulation of Cdk5, thereby explaining the stimulation of dendrite outgrowth by PD98059. Consistently, UV-B (32) and cAMP (36), two potent melanogenic agents that stimulate dendritic growth, were also shown to block cells in G1 phase. The involvement of Cdk5 in the formation of dendrites induced by cAMP-elevating agents or by the MAP kinase pathway inhibition is an appealing hypothesis that needs to be confirmed.

It seems surprising that the activation of MAP kinase leads to the inhibition of melanogenesis, since forskolin and α-MSH, two strong stimulators of melanogenesis, have been shown to activate MAP kinases (10). Hence, cAMP-elevating agents seem to trigger at least two different cascades of molecular events, one ending in the stimulation of melanin synthesis, and a second one decreasing melanogenesis through the activation of the MAP kinase pathway. Induction of antagonistic effects, by a single agent, has already been described in the literature. For instance, EGF activates MAP kinases which phosphorylate the EGF receptor, leading thereby to a decrease in the EGF binding (37). Further, tumor necrosis factor-α induces apoptosis and the activation of NFκB that plays a key protective role against apoptosis (38). We can envision that these opposite effects elicited by one agent can be involved in a fine tuning of a final biological effect. This retrocontrol would avoid overgrowth, overapoptosis, and over-melanin production that could be nocuous for the cell.

In summary, we clearly establish in this report that activation of the MAP kinase pathway is not a required event in the induction of melanogenesis. Furthermore, the activation of this pathway leads to an inhibition of melanoma cell differentiation demonstrating that the up-regulation of melanogenesis by cAMP-elevating agents is the consequence of the activation of two distinct and antagonistic pathways that might allow a precise control of melanocyte and melanoma cell differentiation.

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