Inhibition of the Phosphatidylinositol 3-Kinase/p70<sup>56</sup>-Kinase Pathway Induces B16 Melanoma Cell Differentiation*

(Rosé Buscà‡, Corine Bertolotto, Jean-Paul Ortonne, and Robert Ballotti
From INSERM U385, Faculté de Médecine, 06107 Nice Cedex 02, France)

α-Melanocyte-stimulating hormone and cAMP-elevating agents are known to induce B16 cell differentiation, characterized by increased melanin synthesis and dendrite outgrowth. In order to elucidate intracellular signaling pathways involved in this differentiation process, we focused our interest on the phosphatidylinositol 3-kinase/p70<sup>56</sup>-kinase pathway. The specific inhibition of phosphatidylinositol 3-kinase by LY294002 markedly stimulated dendrite outgrowth, thus mimicking the action of cAMP-elevating agents on B16 cell morphology. In addition, LY294002 and rapamycin, a specific p70<sup>56</sup>-kinase inhibitor, were found to independently stimulate tyrosinase expression, thus increasing melanin synthesis. In an attempt to better dissect the molecular mechanisms triggered by cAMP to induce melanoma cell differentiation, we examined the effects of a cAMP-elevating agent forskolin, on both phosphatidylinositol 3-kinase and p70<sup>56</sup>-kinase activities. Specific kinase assays revealed that forskolin partially inhibited phosphatidylinositol 3-kinase activity and completely blocked p70<sup>56</sup>-kinase activity and phosphorylation. In conclusion, our results clearly demonstrate that the inhibition of phosphatidylinositol 3-kinase and p70<sup>56</sup>-kinase is involved in the regulation of B16 cell differentiation. Furthermore, we provide evidence which suggests that cAMP-induced melanogenesis and dendricity are, at least partially, mediated by the cAMP inhibition of the phosphatidylinositol 3-kinase/p70<sup>56</sup>-kinase signaling pathway.

In the epidermis, melanocytes synthesize melanin, which is responsible for skin pigmentation. Melanin synthesis is carried out by a cell-specific enzymatic pathway controlled by tyrosinase (EC 1.14.18.1), the enzyme that catalyzes the initial two rate-limiting reactions of this process, the hydroxylation of tyrosine to dopa and its subsequent oxidation to dopaquinone (1–4). In vivo, melanogenesis is induced mainly by ultraviolet A and B radiation of sunlight and α-melanocyte-stimulating hormone (α-MSH)<sup>1</sup> (5) which binds to a specific G protein-coupled receptor. In cultured melanocytes or in melanoma cells, melanogenesis can be induced by ultraviolet A and B radiation and by a large array of effectors including α-MSH (4) and pharmacological agents such as forskolin, cholera toxin, or isobutylnymethylxanthine (6–9). These agents increase the intracellular cAMP content, thereby indicating the importance of the cAMP pathway in melanogenesis. The stimulation of melanogenesis by cAMP-elevating agents seems to occur through the induction of tyrosinase expression and stimulation of its intrinsic enzymatic activity ensuing post-translational modifications (10). However, few data are available concerning molecular mechanisms that connect the cAMP signaling pathway and tyrosinase regulation. Recently, we have shown in B16 melanoma cells that cAMP-elevating agents stimulate ERK1 activity and induce its translocation to the nucleus (9), whereas in the majority of cell systems, cAMP has been described to inhibit this kinase (11). Furthermore, concomitantly to the stimulation of ERK1 and melanogenesis, cAMP induces a morphological differentiation characterized by dendrite outgrowth (9) and an inhibition of B16 melanoma cell proliferation. Similar effects including ERK1 activation, neurite outgrowth, and cell growth inhibition have been observed during cAMP-induced differentiation of rat pheochromocytoma PC12 cells (12, 13), which, like melanocytes, are derived from the neural crest. The mechanisms of differentiation in PC12 cells have been thoroughly investigated. Recently, two reports have shown that wortmannin (14), a potent phosphatidylinositol-3-kinase (PI3-K) inhibitor, as well as dominant negative mutants of PI3-K, clearly inhibit nerve growth factor-induced neurite outgrowth in PC12 cells, thus demonstrating a positive involvement of PI3-K in PC12 cell differentiation (15, 16).

PI3-K belongs to a family of signal transducer heterodimeric enzymes composed of a 85-kDa regulatory subunit (p85 α or β) containing SH2 and SH3 domains and a 110-kDa catalytic subunit (p110 α or β) (17, 18) that phosphorylate the D3 hydroxyl in the inositol ring of phosphatidylinositol. PI3-K is activated after association of the p85 regulatory subunit with tyrosine-phosphorylated proteins, including activated tyrosine kinase receptors, non-receptor tyrosine kinases (19–23), and docking proteins such IRS-1 (24). The role of PI3-K in transducing mitogenic signals is currently clearly confirmed, and recently the kinase has been found to be implicated in differentiation (15, 16). Other studies have involved PI3-K in membrane transport and intracellular traffic through the regulation of membrane and cytoskeleton rearrangements occurring in response to growth factor stimulation (14, 25–27). Recent studies have demonstrated that the serine/threonine kinase, p70<sup>56</sup>-kinase (p70<sup>56k</sup>) acts downstream of PI3-K (28, 29). P70<sup>56k</sup> phosphorylates its main target, the 40 S ribosomal cellular signal regulated kinase 1; PNPP, p-nitrophenolphosphate; PI, phosphatidylinositol; PI3-K, phosphatidylinositol 3-kinase; p70<sup>56k</sup>, p70<sup>56</sup>-kinase; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; bp, base pair(s).

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‡ Supported by a postdoctoral fellowship from ARC association (Association pour la Recherche sur le Cancer). To whom correspondence should be addressed; INSERM U385, Faculté de Médecine, Avenue de Valombrose, 06107 Nice Cedex 02, France. Tel.: 33 93 37 77 99; Fax: 33 93 81 14 04; E-mail: busca@unice.fr.

1 The abbreviations used are: α-MSH, α-melanocyte-stimulating hormone; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; ERK1, extracellular signal regulated kinase 1; PNPP, p-nitrophenolphosphate; PI, phosphatidylinositol; PI3-K, phosphatidylinositol 3-kinase; p70<sup>56k</sup>, p70<sup>56</sup>-kinase; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline; bp, base pair(s).
protein S6, which is involved in translational up-regulation of an essential family of mRNAs, including transcripts for ribosomal proteins and elongation factors. Inhibition of the p70\textsubscript{S6K} activity by the immunosuppressant rapamycin or microinjection of neutralizing antibodies severely block cell cycle progression at the middle G\textsubscript{1} phase, indicating that p70\textsubscript{S6K} is necessary for cells to enter the S phase (26).

Taking into account the involvement of the PI3-K pathway in PC12 cell differentiation and in an attempt to search for intracellular signaling pathways involved in melanogenesis, we focused our interest on the implication of PI3-K in the B16 melanoma cell differentiation process. In this report, using the specific PI3-K inhibitor LY294002 (30) and the p70\textsubscript{S6K}-specific inhibitor rapamycin (31), we show that the inhibition of the PI3-K/p70\textsubscript{S6K} pathway mimics the effect of cAMP-elevating agents and leads to a strong stimulation of melanogenesis in B16 melanoma cells. Furthermore, cAMP triggers a significant inhibition of PI3-K activity and a strong blockage of p70\textsubscript{S6K} activity, thus revealing the implication of the PI3-K-p70\textsubscript{S6K} pathway in the transmission of the melanogenic effect of cAMP.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium, antibiotics (penicillin and streptomycin), isobutylmethylxanthine, 12-0-tetradecanoylphorbol-13-acetate, forskolin, bovine serum albumin, protein A-Sepharose, 4-2-aminoethylbenzenesulfon fluoride (AEBSF), aprotinin, leupeptin, i-dopa, benzamidine, PNPP, rapamycin, and phosphatidyl-inositol (PI) were purchased from Sigma. Fetal calf serum was from Life Technologies, Inc. LY294002 was from Biomol Research Laboratories (Plymouth, UK). The culture cell plates and 96-well plates were from Falcon. Polyclonal rabbit antiserum to human tyrosinase (PEP-7) was provided by Dr. V. Hearing (Bethesda, MD), and the secondary fluoroein isothiocyanate-conjugated and peroxidase-conjugated anti-rabbit antibodies were from Dakopatts (Glostrup, Denmark). Antiserum anti-PI3-K was provided by Dr. J. Schlessinger (Rochester, NY). Antibodies to p70\textsubscript{S6K} and ribosomal extracts were kindly given by Dr. G. Thomas (Basel, Switzerland), and antibodies against ERK1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies specific PI3-K inhibitor LY294002 (30) and the p70S6K-specific inhibitor were reverse transcribed using the reverse transcription system from Promega. The CDNA obtained was subjected to 25 cycles of PCR (94°C, 30 s; 55°C, 45 s; 72°C, 1 min) using the following specific primers for the mouse tyrosine kinase gene: 5'-CATTGGTATGTTGAGTCT-3' and 5'-TGGTGTACGTGCTTTGTTCC-3'; and a 1191-base pair PCR product was amplified. Specific primers for the glyceraldehyde-3-phosphate dehydrogenase (Clontech) were added as a control for the same reverse transcriptase product and gave rise to an amplified PCR product of 983 bp.

Preliminary trials showed that, after 25 cycles of PCR, the reaction remained exponential. The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide before visualization using ultraviolet light.

Western Blot Assays—For the tyrosinase immunoblot detection, cells were lysed in phosphate buffer, pH 6.8, containing 1% (w/v) Triton X-100 and 100 IU/ml aprotinin, and 1 mM AEBSF. After vortexing, the extracts were centrifuged at 4°C, 13,000 rpm in an Eppendorf Biofuge for 5 min, and 20 μl of the solubilized proteins (supernatants) were loaded onto 10% SDS-polyacrylamide gels (30:0.8, acrylamide: bisacrylamide). Gels were blotted into nitrocellulose (Amersham Corp.). The nitrocellulose membranes were satu- rated with 5% powdered milk in saline buffer, and tyrosinase was detected with the PEP7 polyclonal antibody at a 1/5000 dilution in the saturation buffer and with a secondary peroxidase-conjugated anti-rabbit antibody at a 1/4000 dilution. After 10-min washes after the primary and secondary antibodies, the membranes were performed using a washing buffer containing 0.5% Triton X-100, 0.5% powdered milk in a saline buffer. The blot was developed with the ECL system from Amersham Corp.

For p70\textsubscript{S6K} detection, we used special 10% polyacrylamide gels (30: 0.1, acrylamide: bisacrylamide) to increase resolution. The rest of the process was performed as described above except that the primary antibody was the M6 polyclonal antibody directed against p70\textsubscript{S6K} (36, 37).

Phosphatidylinositol 3-Kinase Assay—For PI3-K activity assays, cells were extracted in the lysis buffer containing 50 mM Hepes, 150 mM NaCl, 10 mM EDTA, 10 mM Na\textsubscript{3}PO\textsubscript{4}, 100 mM NaF, 2 mM vanadate, 1 mM ATP, 100 μM Trasylol, 1% w/v Triton X-100, pH 7.4, for 15 min at 4°C. The extracts were centrifuged at 13,000 rpm for 10 min at 4°C and were immunoprecipitated with the anti-PI3-K antibody directed against the C-terminal domain of p85 (38) preadsorbed on protein A-Sepharose for 90 min at 4°C under agitation. The immunoprecipitates were washed twice with each of the following buffers: (i) phosphate-buffered saline (pH 7.4) containing 1% Nonidet-P40; (ii) 100 mM Tris, 0.5% LiCl, pH 7.4; and (iii) 10 mM Tris, 100 mM NaCl, pH 7.4, 1 mM EDTA, pH 7.4 (39). The pellets were resuspended in 30 μl of 20 mM Hepes, 0.4 mM EGTA, 0.4 mM Na2HPO\textsubscript{4}, and the kinase reaction was started by addition of phosphatidylinositol (0.2 mg/ml), 10 mM MgCl\textsubscript{2}, and 50 μl γ\textsubscript{32P}-ATP (10 Ci/mmol). After 15 min under slight agitation at room temperature, the reaction was stopped by addition of 15 μl of 4 M HCl, and the phosphoinositides were extracted with 130 μl of chloroform:methanol (1:1). The phospholipids were analyzed by thin layer chromatography and autoradiography (39).

p70\textsubscript{S6K}-Kinase Assay—For p70\textsubscript{S6K} assays, cells were scraped in a buffer containing 50 mM Tris, pH 8.0, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM Na\textsubscript{3}PO\textsubscript{4}, 1% Nonidet-P40, 30 mM PNPP, 0.1 mM AEBSF, 1 mM benzamidine, and 0.5 mM diethiothreitol. Next the
extracts were sonicated in an ice-water bath for 5 min at maximum power and then centrifuged at 13,000 rpm for 5 min at 4 °C. The lysates were incubated with 1 ml of the anti-P70S6K antibody M6 for 1 h. The same amount of protein A-Sepharose (CL4B) was added in each tube followed by another incubation of 20 min at 4 °C under agitation. The immunoprecipitates were washed three times in the lysis buffer and one final time in a buffer containing 50 mM MOPS, pH 7.2, 10 mM PNPP, 0.1 mM AEBSF, 0.1% Triton X-100. The kinase reaction was performed by adding a reaction mix (10 ml per point) containing 1 ml of ribosomal extract prepared as described in Lane et al. (40), cold ATP 30 mM final, and 3 micro Ci of γ-[32P]ATP diluted in the kinase buffer (50 mM MOPS, pH 7.2, 10 mM PNPP, 0.1 mM AEBSF, 1% Triton X-100). The reaction was stopped by adding 10 μl of 2 × Laemmli solution, then the tubes were boiled and centrifuged, and the samples were loaded in a 12.5% polyacrylamide gel and exposed for autoradiography.

RESULTS

Phosphatidylinositol 3-Kinase Inhibition Induces Dendricity in B16 Cells—Since the inhibition of PI3-K activity prevents neurite outgrowth in PC12 cells (15, 16), we first investigated the possible involvement of the PI3-K pathway in the cAMP-induced dendrite outgrowth in B16 melanoma cells. Control and forskolin-treated cells were exposed to PI3-K and p70S6K inhibitors for 48 h, and cell morphology was observed in immunofluorescence experiments using the anti-tyrosinase antibody PEP7 as a primary antibody (Fig. 1). In control conditions, B16 cells displayed a fibroblastic appearance (Fig. 1a) while forskolin treatment promoted the emergence of small and numerous dendrites from the plasma membrane (Fig. 1b). Considering the data reported in PC12 cells, we next wanted to inhibit the cAMP-induced dendrite outgrowth by specifically inhibiting the PI3-K activity with LY294002. The PI3-K inhibitor LY294002 behaves as a competitive inhibitor of the ATP binding site specific for PI3-K and abolishes the activity of this enzyme in vitro and in vivo at low micromolar concentrations but has no effect against PI4-kinase nor a number of intracellular serine/threonine kinases (30, 41). It is worth remarking that LY294002 was chosen because it appears to be a much more stable agent than wortmannin in culture medium (15, 16) (data not shown). Therefore, B16 cells were treated with 15 μM LY294002 alone or simultaneously with 20 μM forskolin and LY294002 for 48 h. Unexpectedly, LY294002 did not inhibit the forskolin-induced dendricity but it rather strengthened the effect of the single forskolin exposure (Fig. 1d). Furthermore, when cells were treated only with LY294002, dendricity was greatly induced, similarly to what is observed upon forskolin stimulation (Fig. 1c). To investigate the implication of the PI3-K signaling pathway in dendrite outgrowth processes, we next looked at the role of p70S6K, which has been reported to constitute an indirect PI3-K target in the same signaling cascade. As shown in Fig. 1f, rapamycin, the specific p70S6K inhibitor, was not able to inhibit the cAMP-induced dendricity, but the kinase inhibitor alone did not induce any apparent dendritogenesis (Fig. 1e). In addition to the information concerning cell dendricity, these immunofluorescence experiments showed that in control conditions (Fig. 1a) or in rapamycin-treated cells (Fig. 1c), tyrosinase was located mainly in the cytoplasm surrounding the nucleus. After forskolin (Fig. 1d) or LY294002 treatment (Fig. 1c), the tyrosinase labeling was found in condensed vesicles which appeared to be the melanosome spreading throughout the dendritic expansions of the membrane. Interestingly, in forskolin-, LY294002-, or rapamycin-treated cells, the immunofluorescence labeling appeared more intense, suggesting that these specific agents increased tyrosinase expression. Previous immunofluorescence experiments using other antibodies directed against tyrosinase con-
PI3-K/p70<sup>S6K</sup> Inhibition and Melanoma Cell Differentiation

Inhibition of PI3-K or p70<sup>S6K</sup> Activities Induces Melanogenesis—To further investigate the effects of PI3-K and p70<sup>S6K</sup> inhibition on melanogenesis, we measured melanin synthesis and tyrosinase activity in B16 cells after the treatment with LY294002 or rapamycin. The color of cell pellets and melanin content quantification using a colorimetric assay are shown in Fig. 2. Forskolin, LY294002, and rapamycin clearly promoted cell darkening. Forskolin and LY294002 induced approximately a 6-fold increase in pigment content, while with rapamycin a 3-fold stimulation was detected. Next we quantified the DOPA oxidase activity of tyrosinase, which constitutes the second reaction catalyzed by the enzyme in the melanin synthesis cascade (Fig. 3). The DOPA oxidase activity was increased approximately 5–6-fold by forskolin treatment. When cells were exposed to the LY294002 PI3-K inhibitor or to the rapamycin p70<sup>S6K</sup> inhibitor, a similar increase in tyrosinase activity was observed.

To determine whether these melanogenic agents might affect directly the tyrosinase expression, tyrosinase mRNA and protein were quantified in control and treated cells. Reverse transcription PCR assays on RNA extracted from control B16 cells using tyrosinase-specific primers produced a 1191-bp fragment corresponding to the tyrosinase mRNA. An increased amount of this PCR fragment was observed with RNA from forskolin, LY294002, and rapamycin treated cells (Fig. 4B), indicating that these agents increased the levels of tyrosinase gene expression compared to nonstimulated cells. A control of PCR amplification, using specific primers for the glyceraldehyde-3-phosphate dehydrogenase transcript, gave similar amounts of a 983-bp PCR product in each condition (Fig. 4A).

Finally, a Western blot detection of tyrosinase was carried out in order to measure the amount of tyrosinase protein in cells exposed to the different agents. Forskolin, LY294002, and rapamycin markedly increased the amount of tyrosinase protein (detected as a band of 70 kDa) compared to the nearly undetectable level of expression in the nonstimulated cells (Fig. 5). This result indicated that the inhibition of PI3-K or p70<sup>S6K</sup> led to a stimulation of tyrosinase expression. The detection of the 44-kDa ERK1 protein ensured that each lane was loaded with the same amount of protein. Taken together these data clearly demonstrate that PI3-K or p70<sup>S6K</sup> inhibition induce an increase of tyrosinase gene expression and amount of protein, thus leading to a stimulation of melanin synthesis.

cAMP-elevating Agents Modulate PI3-K and p70<sup>S6K</sup> Activities—Since PI3-K inhibition by LY294002 and p70<sup>S6K</sup> inhibition by rapamycin appear to trigger the same melanogenic effects as forskolin, we next evaluated the action of this cAMP-elevating agent on PI3-K and P70<sup>S6K</sup> activities. PI3-K was immunoprecipitated from B16 cells and an in vitro phosphorylation assay with a mix of PIs as substrate was performed. Then the phosphatidinositol phosphates were separated by thin layer chromatography and visualized by autoradiography. After treatment of B16 cells with serum for 30 min, we observed a moderate but significant increase in PI phosphorylation (PI-3-P) (Fig. 6). This phosphorylation was completely inhibited in the presence of LY294002 in the kinase reaction. When PI3-K was measured in cells exposed concomitantly to serum and forskolin for 15, 20, or 30 min, we detected a diminished PIs

**Fig. 2.** PI3-K inhibition with LY294002 and p70<sup>S6K</sup> inhibition with rapamycin increase the melanin amount in B16 cells. B16 cells grown in serum containing medium (CONT) or treated either with 20 μM forskolin (FORSK), 15 μM LY294002 (LY), or 1 nM rapamycin (RAPA) were centrifuged to obtain cell pellets that were photographed. Melanin determination was performed after cell lysis as described under “Experimental Procedures.” The total melanin amount is expressed in micrograms of melanin/mg of total protein. Results are means ± S.E. of three independent determinations.

**Fig. 3.** Tyrosinase activity is induced by forskolin, LY294002, and rapamycin. B16 cells grown in serum containing medium (CONTROL) were incubated for 48 h either with 15 μM LY294002 (LY), 1 nM rapamycin (RAPA), or 20 μM forskolin (FORSK) were lysed, and the dopa oxidase activity of tyrosinase was assayed using a solution of 1-dopa as the substrate. Absorbance at 570 nm was read every 10 min as described under “Experimental Procedures,” and the obtained activity was corrected by the amount of protein. Data are means ± S.E. of five experiments performed in triplicate.

**Fig. 4.** Induction of tyrosinase gene expression by LY294002, rapamycin, and forskolin treatments. 5 μg of total RNA from control B16 cells (CONT) or cells treated for 48 h with 20 μM forskolin (FORSK), 15 μM LY294002 (LY), or 1 nM rapamycin (RAPA) were reverse transcribed. The obtained cDNAs were subjected to PCR using specific primers for tyrosinase (TYRO) that gave raise to an amplified product of 1191 bp, and for glyceraldehyde-3-phosphate dehydrogenase as a control that revealed a product of 983 bp (A). Preliminary trials established that after 25 cycles the PCR reaction remained in the exponential phase. The PCR products were electrophoresed on 1% agarose gel and stained with ethidium bromide before visualization using ultraviolet light. Molecular sizes indicated on the left are expressed in base pairs.
p70S6K is activated, it is highly phosphorylated and presents a slower electrophoretic mobility. In contrast, the inactive kinase is less phosphorylated and has a faster electrophoretic migration. We examined the p70\textsuperscript{S6K} electrophoretic mobility in Western blot experiments using the antibody M6 (36, 37). In starved cells, p70\textsuperscript{S6K} appeared as a two principal bands with high electrophoretic mobility representing the unphosphorylated forms of the enzyme. In serum-treated cells we observed two additional bands with higher apparent molecular weights corresponding to the activated p70\textsuperscript{S6K}. This effect of serum was reversed by LY294002 or rapamycin which increased the mobility of the protein. When serum-treated cells were exposed for 15, 20, or 30 min to forskolin, the low electrophoretic mobility forms gradually disappeared, indicating diminished phosphorylation (Fig. 7B). Hence, cAMP inhibited the p70\textsuperscript{S6K} activity as a result of an inhibition of its phosphorylation.

It is worth mentioning that while forskolin induced an increase in the MAP kinase activity in B16 cells as reported (9), neither LY294002 nor rapamycin affected the activity of this kinase measured by in vitro phosphorylation assays (not shown).

**DISCUSSION**

In the present work we provide evidence for the role of the PI3-K/p70\textsuperscript{S6K} pathway in the control of B16 melanoma cell differentiation which is characterized by a stimulation of melanin synthesis and dendrite outgrowth. In PC12 cells, the inhibition of PI3-K activity has been shown to prevent nerve growth factor-induced neurite outgrowth (15, 16). Since PC12 cells share numerous features with B16 cells, we therefore expected that the inhibition of PI3-K would block the cAMP-induced dendrite outgrowth in this melanoma cell line. Strikingly, our studies revealed that, in B16 cells, the inhibition of PI3-K by LY294002 did not block forskolin effects but was sufficient by itself to induce a strong cell dendricity. However, the inhibition of p70\textsuperscript{S6K} that functions downstream PI3-K (29) did not lead to any morphological changes. This observation suggests the existence of PI3-K targets acting upstream or independently of p70\textsuperscript{S6K} that might play a regulatory role in the induction of these morphological modifications. The protein kinase B/Akt which is a serine/threonine protein kinase en-
PI3-K/p70S6K Inhibition and Melanoma Cell Differentiation

PI3-K/p70S6K activity, which has been found to function downstream PI3-K and to participate in the p70S6K activation (44); it is tempting to propose that other unknown targets of protein kinase B/AKT could be involved in the induction of the dendritic phenotype. Similarly, some members of the protein kinase C family such as protein kinase C δ, ε, θ, and ζ, which are directly activated by phosphorylidyinositol phosphate (45–47) are also putative candidates that could participate in this phenomenon. Moreover, it has recently been reported that proteins Rh and Rac, which belong to the superfamily of small G proteins, are indirectly regulated by PI3-K and mediate cytoskeleton rearrangements triggering events such as membrane ruffling and vesicle reorganization (48–51). This points to the putative involvement of these molecules in eliciting dendrite outgrowth processes after cAMP or LY294002 stimulation. Future research is certainly required to identify the pathway leading to this morphological modification (dendriticity) in B16 melanoma cells.

On the other hand, our work demonstrates that the inhibition of PI3-K led to a stimulation of melanin synthesis that appeared to result from an increased tyrosinase activity and expression. In addition, similar effects were observed with rapamycin, indicating that the inhibition of p70S6K is sufficient to induce melanogenesis. However, rapamycin, which is as potent as forskolin or LY294002 in stimulating tyrosinase activity, is markedly less efficient in inducing cell tanning. A possible explanation of this finding might reside in the fact that the synthesis of black melanin (eumelanin) involves other specific regulated enzymes such as tyrosinase-related proteins 1 and 2 (52–54). Thus, we can hypothesize that rapamycin may somehow inhibit the expression or the activity of these tyrosinase-related enzymes or down-regulate other steps downstream of tyrosinase in the melanin production pathway.

It is worth emphasizing that forskolin, LY294002, and rapamycin produced similar effects on melanogenesis. This supports the hypothesis that the induction of melanogenesis by cAMP could be due to a down-regulation of the PI3-K/p70S6K pathway. Indeed an inhibition of PI3-K activity by cAMP has been already described in T lymphocytes (55) and in neutrophils (56). Furthermore, cAMP-elevating agents have been shown to inhibit p70S6K activity in Swiss 3T3 cells (57) and in T lymphocytes (55). In B16 melanoma cells, forskolin partially inhibits PI3-K activity and completely blocks p70S6K activity. In addition, this cAMP-elevating agent as well as α-MSH (not shown), a physiological melanogenic agent that increases the intracellular cAMP levels, induced a dramatic inhibition of p70S6K phosphorylation which constitutes an essential event for the activation of the kinase. Since the inhibition of PI3-K activity by cAMP appears rather weak, this could not entirely explain the strong inhibition of p70S6K activity found after the treatment with cAMP elevating agents. Therefore it can be proposed that cAMP could act through the regulation of the activity of protein kinase B or other kinases involved in phosphorylation and activation of p70S6K.

Interestingly, we observed a significant diminution in the cell number and in DNA synthesis, without cytotoxic effects, after forskolin, LY294002, or rapamycin treatments (not shown), revealing an arrest of cell growth. This could be due to the inhibitory effects of forskolin, LY294002, or rapamycin on p70S6K activity, which has been found essential for progression through the G1 phase of the cell cycle (40). In contrast, serum or growth factors, such as 12-O-tetradecanoylphorbol-13-acetate or epidermal growth factor that activate p70S6K, display an inhibitory effect on melanogenesis in B16 cells (not shown). These findings suggest that the induction of melanogenesis and morphological differentiation in B16 cells might require the arrest of cell growth at the G1 phase. A similar conclusion has been reached concerning the induction of neurite outgrowth in the neuroblastoma cell line NE1-115 (58).

The precise molecular mechanisms that connect the inhibition of the PI3-K/p70S6K pathway to the induction of melanogenesis and dendrite outgrowth remain to be elucidated. Nevertheless, the existence of a nuclear isoform of the S6 kinase (p58S6K) (59) is consistent with the idea that this kinase could modulate the transcription of transcription factors. Indeed, cAMP-responsive element modulator τ has been reported to be phosphorylated and activated by p70S6K (60). On the other hand, microphthalmia, a tissue-specific transcription factor that has been recently shown to play a role in the stimulation of tyrosinase gene expression by cAMP (61), could also be a target of p70S6K. Thus the inhibition of this kinase by cAMP would lead to a decreased activation of microphthalmia, or other transcription factors involved in B16 cell differentiation, and thereby regulate their transcriptional activities.

Taken together our results demonstrate the involvement of the PI3-K/p70S6K pathway in B16 melanoma cells differentiation and indicate for the first time that the inhibition of this signaling cascade by cAMP is likely to be a key event for the cAMP-induced melanogenesis and dendrite outgrowth. Furthermore, the novel finding that the inhibition of the PI3-K/p70S6K pathway displays a positive effect on B16 melanoma cell differentiation whereas the PI3-K inhibition blocks the differentiation process in PC12 cells, reveals that the study of several cell systems is required to fully understand the mechanisms of cell differentiation.

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