Microphthalmia Associated Transcription Factor Is a Target of the Phosphatidylinositol-3-Kinase Pathway

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In B16 melanoma cells, cyclic adenosine monophosphate inhibits the phosphatidylinositol-3-kinase and the phosphatidylinositol-3-kinase inhibitor, LY294002, stimulates melanogenesis. However, the molecular mechanisms, by which phosphatidylinositol-3-kinase inhibition increases melanogenesis remained to be identified. In this study, we show that LY294002 up-regulates the expression of the melanogenic enzymes, tyrosinase and Tyrp1, through a transcriptional mechanism that involves microphthalmia associated transcription factor, a basic helix-loop-helix transcription factor, which plays a key role in melanocyte survival and differentiation. Further, we observe that LY294002 increases the intracellular content of microphthalmia associated transcription factor, thereby demonstrating that microphthalmia associated transcription factor is also a convergence point of the phosphatidylinositol-3-kinase signaling pathway. Finally, our results indicate that LY294002 controls microphthalmia associated transcription factor at the transcriptional level through distal regulatory element that remain to be identified. Interestingly, we have recently reported that cAMP-elevating agents, through a phosphatidylinositol-3-kinase/AKT inhibition and a glycogen synthase kinase 3β activation, may stimulate microphthalmia associated transcription factor binding to its target sequence, suggesting that inhibition of the phosphatidylinositol-3-kinase is implicated in the stimulation of melanogenesis at different levels. Thus, the results presented in this report strengthen the importance of the phosphatidylinositol-3-kinase pathway in the regulation of melanogenesis and emphasize the complexity of the cyclic adenosine monophosphate signaling that controls melanocyte differentiation and melanogenesis. ***Key words: cyclic adenosine monophosphate/microphthalmia associated transcription factor/phosphatidylinositol-3-kinase/tyrosinase/TYRP1. J Invest Dermatol 121:831–836, 2003***

In mammals, pigmentation results from the synthesis and distribution of melanin in the skin, hair bulbs, and eyes. Melanin synthesis or melanogenesis occurs in melanocytes through an enzymatic process, catalyzed by tyrosinase and tyrosinase-related protein 1 (Tyrp1), which converts tyrosine to melanin pigments (Hearing, 1987; Prota, 1988; Jimenez-Cervantes *et al*, 1994; Kobayashi *et al*, 1994). *In vivo*, these pigments play a crucial photoprotective role against the carcinogenous effects of ultraviolet radiation of the solar light.

Compelling evidence has demonstrated the key role of the melanotropic hormone α-melanocyte-stimulating hormone and adrenocorticotropic hormone in the control of pigmentation. α-melanocyte-stimulating hormone and adrenocorticotropic hormone bind to the Gαs-coupled MC1 receptor (MC1R) that leads to adenylate cyclase activation, elevation of the intracellular cyclic adenosine monophosphate (cAMP) content, and activation of protein kinase A (PKA). Patients in which the cAMP/PKA pathway has been altered, present skin pigmentation defects, thereby demonstrating the importance of the cAMP/PKA pathway in the regulation of melanogenesis (Schwindinger *et al*, 1992; Kirschner *et al*, 2000). *In vitro*, the melanogenic effects of α-melanocyte-stimulating hormone can be mimicked by pharmacologic agents such as forskolin, a direct activator of adenylate cyclase (Englaro *et al*, 1995). We have previously shown that cAMP-elevating agents increase the expression of the melanogenic enzymes, tyrosinase and Tyrp1, by stimulating the transcription of their cognate genes. We also reported that the M-box (AGTCATGTGCT), a highly conserved DNA sequence, identified in the promoter of these enzymes, is essential for their cAMP responsiveness. The M-box binds MITF (microphthalmia-associated transcription factor), a transcription factor of the basic-helix-loop-helix-leucine-zipper family (b-HLH-LZ) that plays a crucial part in melanocyte development (Hodgkinson *et al*, 1993; Steingrimsson *et al*, 1994). In the mouse, mutations at the *mi* locus lead to coat color dilution, white spotting, or complete loss of pigmentation due to absence of melanocyte (Hodgkinson *et al*, 1993; Hughes *et al*, 1993). Similarly, in humans, mutations in *MITF* have been linked to abnormal pigmentation observed in Waardenburg syndrome type IIa (Hughes *et al*, 1994; Tassabehji *et al*, 1994). Interestingly, the cAMP/PKA pathway, through phosphorylation and activation of the cAMP response element binding protein (CREB) transcription factor, upregulates the *Mitf* promoter activity, thereby leading to stimulation of MITF expression.

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Abbreviations: CREB, cyclic-AMP response element binding protein; fsk, forskolin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3β, glycogen synthase kinase 3 β; MITF, microphthalmia-associated transcription factor; PKA, protein kinase A, PI3K, phosphatidyl-inositol-3-kinase.

Further, we have previously reported that Mitf is absolutely required to mediate the melanogenic effects of cAMP (Bertolotto *et al*, 1998a), indicating that Mitf is also crucial for melanocyte differentiation and melanogenesis.

Focusing our interest on the molecular mechanisms involved in the regulation of melanin synthesis by cAMP, we have already reported that cAMP promotes an inhibition of the phosphatidylinositol-3 kinase (PI3K) activity (Busca *et al*, 1996). PI3K is a lipid kinase that has been implicated in several physiologic processes, such as proliferation, survival, intracellular traffic, and cell differentiation (Katso *et al*, 2001). Additionally, a specific inhibitor of PI3K, LY294002, mimics the effects of cAMP leading to the induction of melanogenesis and melanocyte differentiation (Busca *et al*, 1996). These observations suggest that inhibition of PI3K is an important step of cAMP-induced pigment production.

In this study, we have investigated how the inhibition of PI3K promotes an induction of melanogenesis. First, we show that the inhibitor of PI3K, LY294002, increases the expression of tyrosinase and Tyrp1 through a transcriptional mechanism. Further, a dominant negative form of Mitf strongly reduces the stimulation by LY294002 of the *tyrosinase* and *Tyrp1* promoter activities, demonstrating the involvement of Mitf in the regulation of melanogenic enzyme transcription by LY294002. We next observed an increased Mitf binding to the M-box sequence of the *tyrosinase* and *Tyrp1* promoters following LY294002 treatment, which results from a stimulation of Mitf expression. Finally, our results indicate that elevation of Mitf levels by LY294002 is regulated at the transcriptional level through distal regulatory elements that remain to be identified.

MATERIALS AND METHODS

Materials Forskolin, sodium fluoride, sodium orthovanadate, 4-(2-aminoethyl)-benzene-sulfonyl fluoride (AEBSF), aprotinin, and leupeptin were purchased from Sigma (Sigma Chem Co. St. Louis, MO). LY294002 was from MERCK Eurolab (Darmstadt, Germany). Dulbecco's modified Eagle's medium, trypsin, and lipofectamine reagent were from Invitrogen (San Diego, CA) and fetal bovine serum was from Hyclone (Logan, UT). All clinical studies were approved by the IRB for animal use and patient consent forms were signed.

Antibodies The monoclonal anti-MITF antibody was from Dr D. Fisher (Boston, Massachusetts). The rabbit polyclonal anti-tyrosinase (pep 7) and anti-Tyrp1 (pep1) antibodies were from Dr V. Hearing (Bethesda, Maryland). The polyclonal phospho-specific AKT (S473), CREB (S133), p42/44 mitogen-activated protein kinase (Thr202/Tyr204), and β-catenin (S33/S37/T41) antibodies were from Cell Signaling (Cell Signaling Technology, Beverly, MA) and the monoclonal ERK2 (D-2) antibody was from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA). The phospho-glycogen synthase antibody (Ab-1) and the phospho-specific Tau antibody (S396) were purchased from Oncogene Research Products (Darmstadt, Germany). Horseradish peroxidase or fluorescein isothiocyanate-conjugated anti-rabbit or anti-mouse antibodies were from Dakopatts (Glostrup, Denmark).

Methods

Cell cultures B16/F10 murine melanoma cells were grown at 37°C under 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 7% fetal bovine serum and penicillin (100 U per mL)/streptomycin (50 μg per mL).

Melanin content determination B16 were seeded in six-well dishes and exposed to 20 μM forskolin, or LY294002 for the concentration and time indicated in the figure legends. Then, melanins were solubilized in 0.5 M NaOH for 1 h at 65°C. Absorbance at 405 nm was compared with a standard curve of known concentrations of fungal melanin prepared in a final NaOH concentration of 0.5 M. The melanin content was corrected by the total cell number of the dish and expressed as fold stimulation of melanin of control cells.

Western blot assays B16 melanoma cells were grown in six-well dishes with forskolin or LY294002. Cells were lyzed in buffer containing 50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 μM leupeptin, 1 mM AEBSF, 100 U per mL aprotinin, 10 mM NaF, and 1 mM Na_3VO_4. Samples (30 μg) were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and then exposed to the appropriate antibodies. Proteins were visualized with the enhanced chemiluminescence system from Amersham using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody.

Expression vectors, transfection, and luciferase assays The luciferase reporter plasmids pTyro, pTyrp1, and pMITF and the expression vector encoding the wild-type or the dominant negative form of MITF were previously described (Bertolotto *et al*, 1998a, c). TOPFlash luciferase reporter plasmid, containing 3 Lef/TCF binding site cloned upstream of the c-fos promoter, was a kind gift of Dr L. Larue (Orsay, France).

Briefly, B16 melanoma cells were seeded in 24-well dishes and transient transfections were performed the following day using 2 μL of lipofectamine and 0.5 μg of total DNA plasmid. pCMVβGal was transfected with the test plasmids to control the variability in transfection efficiency. After 48 h, cells were harvested in 50 μL of lysis buffer and assayed for luciferase and β-galactosidase activities. All transfections were repeated at least three times and performed in triplicate.

Northern blot analysis Poly(A)$^+$ RNA were isolated from control and forskolin or LY294002-treated cells using the mRNA purification kit from Qiagen Inc., Valencia, CA. (OligotexTM, mRNA) and total RNA was isolated by a modification of the method of Chomczynski and Sacchi (1987). RNA was denatured for 5 min at 65°C in a formamide/formaldehyde mixture, separated by electrophoresis in a 1% agarose/7% formaldehyde gel, transferred to a nylon membrane (Hybond N, Amersham Bio Sciences, Orsay, France) in 20 × sodium citrate/chloride buffer (3 M NaCl, 0.3 M sodium citrate pH 7) and hybridized to MITF and GAPDH probe labeling by random priming with α-[^{32}P] deoxycytidine triphosphate (Amersham).

Immunofluorescence studies B16 melanoma cells were grown on glass coverslips (2×10^4 cells per point) in 12-well dishes and treated for 6 h with 20 μM forskolin or 10 μM LY294002. Cells were then washed, fixed at room temperature for 20 min with 3% paraformaldehyde, and permeabilized by a 2 min treatment with phosphate-buffered saline 1% Triton before being exposed to an anti-MITF antibody for 1 h at room temperature. Cells were next incubated with FITC coupled anti-mouse antibody for 1 h at room temperature and the cells were washed with phosphate-buffered saline in which bisbenzidine (0.5 μg per mL) was added. Finally, coverslips were mounted in moviol immunofluorescence mounting medium and examined with the 40 × objective using Zeiss Axiophot microscope equipped with epifluorescence illumination.

Nuclear extracts and gel mobility shift assay Nuclear extracts from control cells or cells incubated with 20 μM forskolin or 10 μM LY294002 were prepared as previously described. Double-stranded synthetic M-box, 5′-GAAAAAGTCATGTGCTTTGCAGAAGA-3′ was γ^{32}P end-labeled using T4 polynucleotide kinase. Five micrograms of nuclear proteins were preincubated in a binding buffer containing 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM dithiothreitol, 1 mM ethylenediamine tetraacetic acid, 4% glycerol, 80 μg per mL of salmon sperm DNA, 0.1 μg poly(dIdC), 10% fetal bovine serum, 2 mM $MgCl_2$, and 2 mM spermidine for 15 min on ice. Then, 30,000 to 50,000 cpm of ^{32}P-labeled probe were added to the binding reaction for 10 min at room temperature. DNA-protein complexes were resolved by electrophoresis on a 4% polyacrylamide gel (37.5:1 acrylamide/bisacrylamide) in TBE buffer (22.5 mM Tris-borate, 0.5 mM ethylenediamine tetraacetic acid, pH 8) for 90 min at 100 V. For supershift assays, 0.3 μL of preimmune serum or anti-MITF antibody were preincubated with nuclear extracts in the binding reaction buffer before adding the labeled probe.

RESULTS

LY294002 increases tyrosinase and Tyrp1 expression through a transcriptional mechanism In this study, we investigated the molecular mechanisms by which the inhibition of the PI3K pathway stimulates melanogenesis. With this aim we used LY294002, a pharmacologic inhibitor of PI3K that binds to the adenosine triphosphate binding site of PI3K and specifically blocks the activity of this kinase. In **Fig 1(*A*)**, we showed that LY294002 inhibited the phosphorylation of AKT that is a direct target of PI3K; however, LY294002 did not affect the phosphorylation of p42/44 mitogen-activated protein kinase or

CREB transcription factor. These results indicate that LY294002 specifically inhibits the PI3K signaling but has no effect on the p42/44 mitogen-activated protein kinase or PKA pathways. Dose-response and time course experiments revealed a maximal induction of melanin synthesis after 48 h of cell exposure to 10 μM of LY294002 (**Fig 1*B***). Melanin synthesis assays were corrected by the total cell number as forskolin and LY294002 decreased cell growth by about 22% ± 1% and 30% ± 3%, respectively. Next, dose-response and time course assays of the LY294002 effects on tyrosinase and Tyrp1 were performed. Consistent with previous observations, western blot experiments revealed a maximal stimulation of tyrosinase and Tyrp1 expression after cell treatment with 10 μM of LY294002 for 48 h (**Fig 1*C***). These observations were not due to differences in loading as shown by ERK2 detection. Then, plasmids containing a fragment of the *tyrosinase* or *Tyrp1* promoter, cloned upstream of the luciferase reporter gene, were transiently transfected in B16 cells. Cells were incubated with LY294002, and luciferase activity, reflecting the activity of the promoters, was measured. We observed that LY294002 induced a 5- and 4-fold stimulation of the transcriptional activity of the *tyrosinase* and *Tyrp1* promoters, respectively (**Fig 1*D***). As a control, we used forskolin, which has been described to increase the expression of tyrosinase and Tyrp1 through a transcriptional mechanism (**Fig 1*B,C***). Taken together, our results show that inhibition of PI3K upregulates the transcriptional activity of the *tyrosinase* and *Tyrp1* promoters, thereby leading to stimulation of tyrosinase and Tyrp1 expression and induction of melanogenesis.

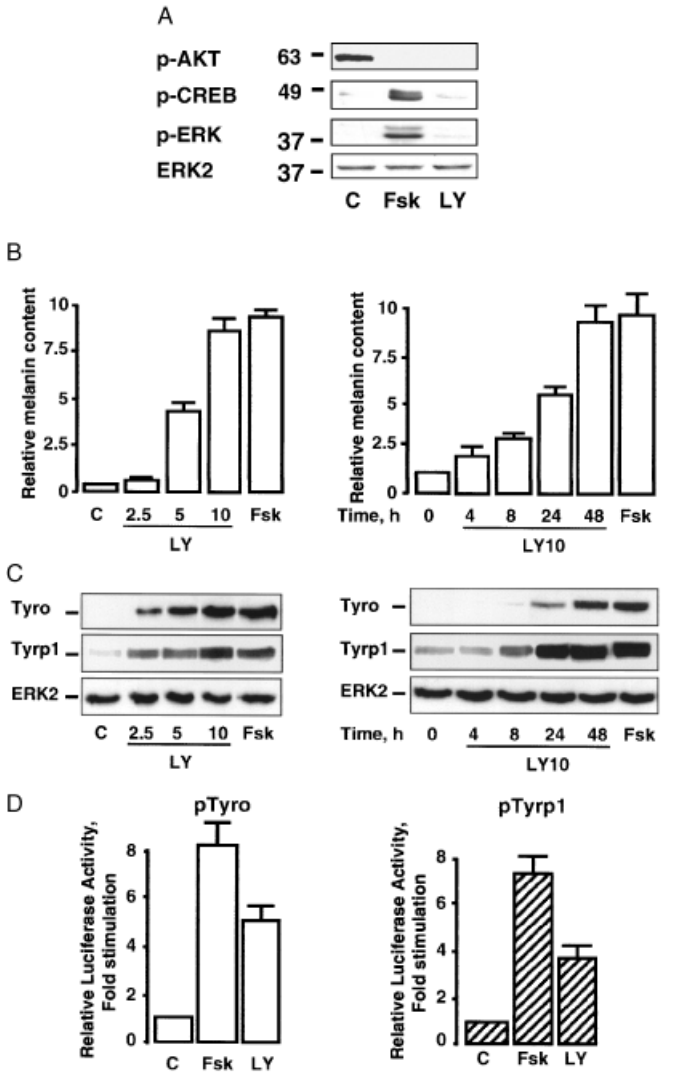

Figure 1. LY294002 increases tyrosinase and Tyrp1 expression by regulating their promoter activities. (*A*) Thirty micrograms of protein extracts from unstimulated cells or cells incubated with LY294002 for 1 h were submitted to immunoblotting using phospho-specific AKT (p-AKT), phospho-specific CREB (p-CREB), phospho-specific ERK (p-ERK) antibodies, or antibody that recognizes total ERK2. Molecular masses, indicated on the left, are expressed in kilodaltons. (*B*) Melanin content from control B16 melanoma cells or B16 cells exposed to 20 μM forskolin (Fsk) or LY294002 (LY) at the concentration and times indicated on the figure, was determined as described in *Materials and Methods*. Results are expressed as fold stimulation of melanin detected in control cells. Results are mean ± SEM of three independent experiments. (*C*) Cells were treated as in (*B*) and then 30 μg of protein extracts were subjected to western blot analysis using the pep7 and pep1 antibodies for, respectively, tyrosinase and Tyrp1 detection. Even loading of each lane was ensured by ERK2 detection. (*D*) B16 cells were transiently transfected with 0.3 μg of the luciferase reporter plasmid pTyr or pTyrp1 and cells were left untreated or treated for 48 h with 20 μM forskolin (Fsk) or 10 μM LY294002 (LY). Then, luciferase activity was measured and the results are expressed as fold stimulation of the basal luciferase activity from control cells. Data are mean ± SEM of three experiments performed in triplicate.

MITF is a key effector of LY294002-induced melanogenesis

Mitf, that plays a central role in melanocyte fate and differentiation, has been demonstrated to be an essential signal transducer in cAMP-induced melanogenesis. We thus studied the involvement of this transcription factor in the regulation of melanogenesis by LY294002. The effect of a dominant negative form of Mitf, lacking the N-terminal transactivation domain (Mi-ΔNT), was assayed on the response of the *tyrosinase* and *Tyrp1* promoters to LY294002. We showed that the Mi-ΔNT transfection strongly reduced the sensitivity of the *tyrosinase* and *Tyrp1* promoters to LY294002, which indicates the important role of Mitf in LY294002-induced melanogenic gene expression (**Fig 2*A***). We next performed gel shift experiments using as labeled probe the M-box, which is the target sequence of Mitf in the *tyrosinase* and *Tyrp1* promoters, respectively (**Fig 2*B***). Nuclear extracts from control cells formed complexes with the labeled M-box (*lane 1*), that were greatly increased when reactions were

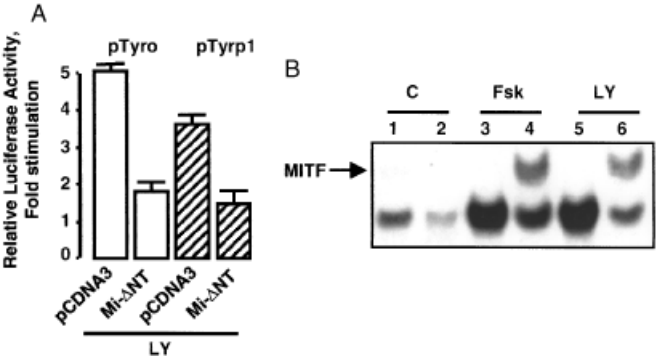

Figure 2. MITF mediates the effect of LY294002 on tyrosinase and Tyrp1 promoter activities. (*A*) B16 cells were transiently transfected with 0.3 μg of the luciferase reporter plasmid pTyro or pTyrp1 with or without 0.05 μg of $pCDNA_3$ encoding Mi-ΔNT. Cells were exposed to LY294002 10 μM for 48 h. Luciferase activity was normalized by the β-galactosidase activity and the results were expressed as fold stimulation of the basal luciferase activity from control cells. Data are mean ± SEM of three experiments performed in triplicate. (*B*) Gel shift assays were designed using the ^{32}P-labeled M-box and B16 nuclear extracts from control cells or cells exposed 4 h to 20 μM forskolin (Fsk) or 10 μM LY294002 (LY). In *lanes 1, 3, and 5*, 0.3 μL of preimmune serum and in *lanes 2, 4, and 6*, 0.3 μL of a specific anti-microphthalmia serum was added to the reaction.

performed with nuclear extracts from forskolin (*lane 3*) or LY294002 (*lane 5*) treated cells. These complexes were strongly displaced by a monoclonal anti-MITF antibody (*lanes 2, 4, and 6*), demonstrating the specific interaction of Mitf with the labeled M-box. Together, our results indicate that the LY294002-increased Mitf binding to its target sequence leads to a stimulation of *tyrosinase* and *Tyrp1* gene expression.

LY294002 upregulates the expression of MITF Immunofluorescence studies with the monoclonal anti-MITF antibody showed that forskolin or LY294002 increased the level of Mitf compared with nontreated cells (**Fig 3*A***). On the other hand, no change in Mitf cellular localization could be detected. Phase contrast microscopy analysis showed, as previously described, that forskolin or LY294002 dramatically increased B16 melanoma cell dendricity. Next, western blot experiments using the monoclonal anti-MITF antibody were carried out to determine the effects of PI3K inhibition on Mitf expression. Mitf, appeared as a doublet in control cells, which was increased after LY294002 exposure (**Fig 3*B***). Stimulation of Mitf expression by LY294002 or forskolin reached its maximum levels after 4 h to 8 h of treatment. Noteworthy, stimulation of Mitf expression by forskolin appeared more sustained than that evoked by LY294002. Finally, we investigated how LY294002 upregulated Mitf expression. Northern blot experiments revealed that LY294002, like forskolin, increased *Mitf* mRNA levels (**Fig 4*A***, *upper panel*). The equal loading of each lane was shown by GAPDH detection (**Fig 4*A***, *lower panel*). Further, the effect of actinomycin D, an inhibitor of the transcriptional processes, was assayed on the regulation of *Mitf* mRNA by forskolin and LY294002. We observed that incubation of control B16 melanoma cells with actinomycin D decreased *Mitf* mRNA below the basal level. As expected, actinomycin D completely abolished the induction of *Mitf* mRNA by forskolin (**Fig 4*B***, *upper panel*). Additionally, actinomycin D totally blocked the stimulation of the *Mitf* mRNA evoked by LY294002, indicating that LY294002 as well as forskolin stimulate *Mitf* gene transcription rather than *Mitf* messenger stability. These observations were not due to a difference in loading as shown by the bromide ethidium staining of the 28S and 18S RNA (**Fig 4*B***, *lower panel*). In conclusion, these results demonstrate that LY294002 increases *Mitf* mRNA through a transcriptional process.

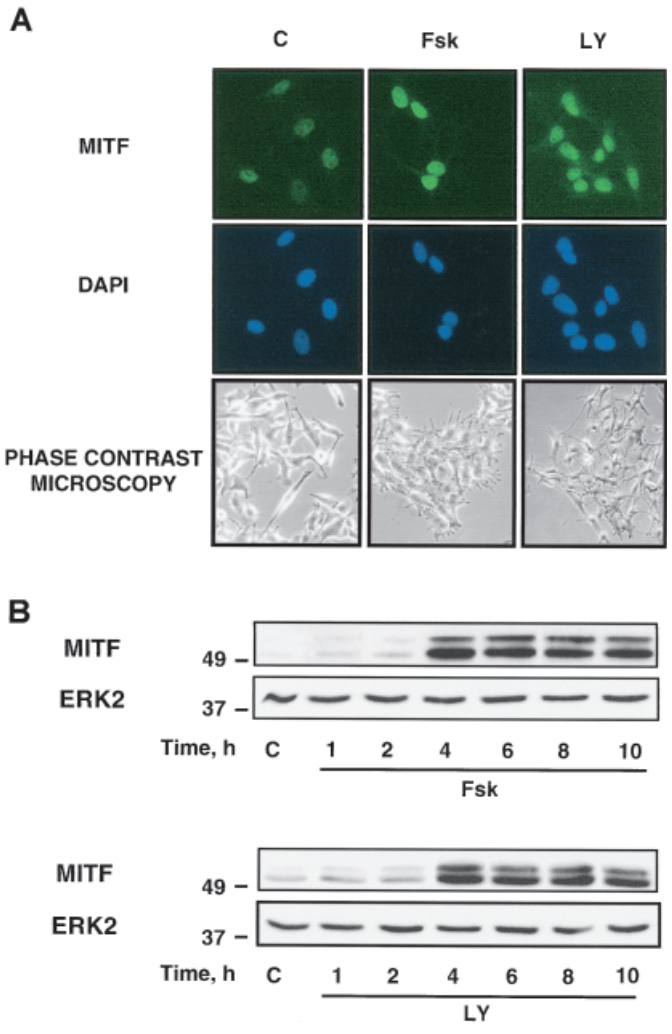

Figure 3. LY294002 increases MITF expression. (*A*) Immunofluorescence labeling of unstimulated cells or cells treated with 20 μM forskolin (Fsk) or to 10 μM LY294002 (LY) was designed with the monoclonal anti-MITF antibody. (*B*) Thirty micrograms of protein extracts from control cells or cells incubated with 20 μM forskolin (Fsk) or 10 μM LY294002 (LY) for the indicated times were submitted to western blot analysis using a monoclonal anti-MITF antibody. Molecular masses, indicated on the left, are expressed in kilodaltons.

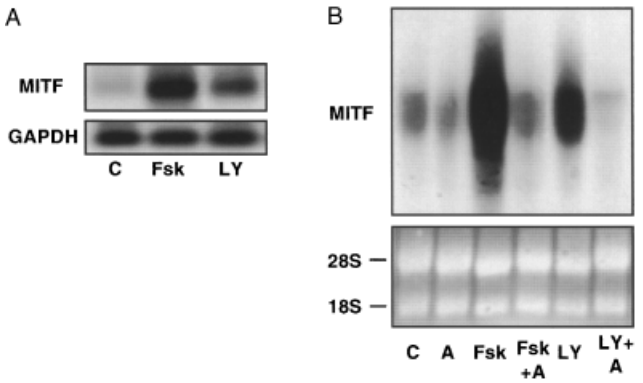

Figure 4. LY294002 controls MITF transcription. (*A*) mRNA from control B16 cells or cells exposed for 2 h to 20 μM forskolin or 10 μM LY294002 were analyzed by Northern blot using MITF and GAPDH probes. (*B*) B16 cells were left untreated or were incubated with actinomycin D 2.5 μg per mL before adding 20 μM forskolin or 10 μM LY294002 for 2 h. Then, total mRNA was extracted and analyzed by Northern blot using MITF and GAPDH probes.

LY294002 does not activate the 2.1 kb fragment of the MITF promoter cAMP elevating agents upregulate the activity of the *Mitf* promoter through phosphorylation and activation of the CREB transcription factor. As shown in **Fig 1**(***A***) LY294002 did not promote CREB phosphorylation. Further, we have recently demonstrated that LY294002 and forskolin lead to the activation of the downstream AKT target, glycogen synthase kinase 3 β (GSK3β). Then, activated GSK3β phosphorylates its substrates such as β-catenin, glycogen synthase, and tau (**Fig 5*A,B***). β-catenin, is one of the factors that controls *Mitf* transcription. LY294002, however, did not affect the intracellular levels of β-catenin or the activity of a TOPFlash reporter plasmid. These observations preclude a possible involvement of β-catenin/Lef complex in *Mitf* activation by LY294002 (**Fig 5*A***). Next, B16 cells were transiently transfected with a reporter plasmid containing a 2.1 kb fragment of the *Mitf* promoter upstream of the luciferase gene and then were incubated with LY294002 (**Fig 5*C***). A time course experiment indicated that LY294002 had no effect on the activity of the *Mitf* promoter, compared with forskolin, which caused, as previously described, a 7-fold stimulation of this promoter activity. These observations indicate that LY294002 does not act on the proximal region of the *Mitf*

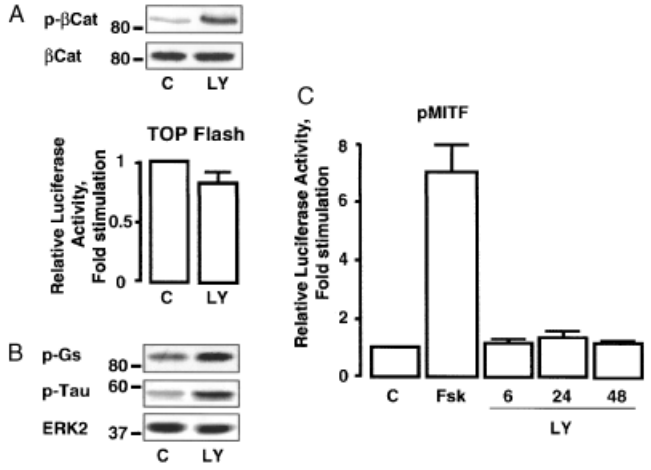

Figure 5. Involvement of distal regulatory element in the response to LY294002 of the MITF promoter. (*A*) Thirty micrograms of protein samples from B16 melanoma cells nonstimulated or incubated for 1 h with 20 μM forskolin (Fsk) or 15 μM LY294002 (LY) were subjected to western blot analysis using a phospho-specific β-catenin (p-β-cat) antibody or an antibody that recognizes total β-catenin (β-cat). B16 cells were transiently transfected with a TOPFlash reporter plasmid and exposed to LY294002 48 h. Luciferase activity was normalized by the β-galactosidase activity and the results were expressed as fold stimulation of the basal luciferase activity from control cells. Data are mean ± SEM of three experiments performed in triplicate. (*B*) Protein samples of cells treated as in (*A*) were submitted to immunoblotting using phospho-specific glycogen synthase (p-GS) or phospho-specific Tau (p-Tau) antibodies or antibody that recognizes ERK2 independently of its phosphorylation state. Molecular masses, indicated on the left, are expressed in kilodaltons. (*C*) B16 cells were transfected with 0.3 μg of the luciferase reporter plasmid pMITF and then exposed to 20 μM forskolin (Fsk) for 48 h or to 10 μM LY294002 (LY) for the indicated times. Luciferase activity was normalized by the β-galactosidase activity and the results were expressed as fold stimulation of the basal luciferase activity from unstimulated cells. Data are mean ± SEM of three experiments performed in triplicate.

promoter and, suggest that LY294002 regulates *Mitf* transcription through distal regulatory elements.

DISCUSSION

In a previous study, it was shown that cAMP, a potent inducer of melanogenesis, promotes an inhibition of the PI3K, and that LY294002, a specific inhibitor of PI3K, increases melanin synthesis (Busca *et al*, 1996). To investigate how inhibition of PI3K stimulates pigment production, we studied the effects of LY294002 on different parameters of melanin synthesis.

In this study, we first show that LY294002, by regulating the activity of the *tyrosinase* and *Tyrp1* promoter activities leads to an increase in tyrosinase and Tyrp1 expression and stimulation of melanogenesis.

Further, we demonstrate that Mitf, a transcription factor that plays a key role in melanocyte survival and differentiation (Hodgkinson *et al*, 1993; Steingrimsson *et al*, 1994; McGill *et al*, 2002) is implicated in the responsiveness of the melanogenic gene promoters to LY294002. Finally, our results indicate that LY294002 stimulates Mitf expression through a transcriptional mechanism that involves distal regulatory elements. Indeed, Northern blot experiments revealed that LY294002 increases *Mitf* mRNA levels in B16 melanoma cells. Further, actinomycin D, an inhibitor of transcription, prevents LY294002-induced upregulation of *Mitf* mRNA, suggesting that LY294002 regulates Mitf expression at the transcriptional level. To understand how LY294002 controls *Mitf* transcription, we focused our attention on the CREB transcription factor. First, we have previously reported that phosphorylation and activation of CREB transcription factor by PKA stimulates Mitf expression (Bertolotto *et al*, 1998a). Secondly, AKT, a downstream target of PI3K, has been shown to promote the phosphorylation and activation of the phosphodiesterase 3B (PDE3B), which down-modulates the intracellular cAMP content (Kitamura *et al*, 1999). Thus, it was tempting to hypothesize that LY294002, through the inhibition of the PI3K/AKT/PDE3B cascade, maintained high intracellular cAMP levels, thereby leading to PKA activation, CREB phosphorylation, and stimulation of the *Mitf* promoter activity; however, we show that LY294002 does not affect CREB phosphorylation, demonstrating that CREB is not implicated in the regulation of *Mitf* transcription by LY294002. Next, we focused our attention on β-catenin, which has been involved in *Mitf* activation through its interaction with the transcription factors of the Lef/TCF family. We observed that LY294002 induces the phosphorylation of β-catenin; however, LY294002 does not significantly affect the activity of a TOPFlash reporter plasmid that is controlled by the β-catenin/Lef/TCF complex, meaning that phosphorylation of β-catenin by LY294002 is not sufficient to induce its degradation. We thus hypothesized that, LY294002 could trigger another regulatory element in the *Mitf* promoter. Sox10 and Pax3 transcription factors have been shown to play an important part in *Mitf* promoter activity, indicating that these factors could be potential targets of LY294002 action (Watanabe *et al*, 1998; Takeda *et al*, 2000b; Verastegui *et al*, 2000). A 2.1 kb fragment of the *Mitf* promoter containing such regulatory sequences, however, was not stimulated by LY294002, demonstrating that the proximal regulatory region does not mediate the response of the *Mitf* promoter to LY294002. Recently, a distal regulatory region located 14.5 kb upstream from exon 1 of the human MITF gene has been shown to enhance the activity of the *Mitf* promoter (Watanabe *et al*, 2002). These data are in agreement with the presence of crucial regulatory elements upstream of the 2.1 kb fragment of the *Mitf* promoter and suggest that these sequences could mediate the sensitivity of the *Mitf* promoter to LY294002. Mitf has been shown to be the target of different signaling pathways that regulate pigment production (Bertolotto *et al*, 1998a, b; Price *et al*, 1998; Kim *et al*, 2002). Interestingly, our results demonstrate that Mitf is also a convergence point of the PI3K pathway, thus reinforcing the key role of Mitf in melanocyte differentiation and melanogenesis. Furthermore, we have recently shown that cAMP, through AKT inhibition, activates the GSK3β, and inhibition of GSK3β by lithium decreases forskolin-induced tyrosinase promoter activity (Khaled *et al*, 2002). In agreement with the report from Takeda *et al* (2000a), we proposed that activation of GSK3β by cAMP would lead to phosphorylation of Mitf, thus increasing its binding to the M-box sequence in the *tyrosinase* and *Tyrp1* promoters, respectively. Therefore, the PI3K pathway stimulates melanogenesis by at least two mechanisms. First, cAMP, through inhibition of PI3K and AKT, and activation of GSK3β, stimulates Mitf binding to the M-box of the *tyrosinase* promoter, increasing the melanogenic enzyme expression (Khaled *et al*, 2002). Second, LY294002 would control the activity of the *Mitf* promoter through distal regulatory elements that remain to be identified.

Interestingly, forskolin gives rise to a more important and sustained expression of Mitf compared with the LY294002 treatment. Taken together, our results suggest that forskolin mediates its effects through the combined activation of the PKA/CREB pathway and inhibition of PI3K, whereas LY294002 action is only the result of PI3K inhibition. The report from Khaled *et al* (2002), mentioned that cAMP inhibits AKT in a PKA-independent manner reinforcing the notion that PKA and PI3K regulate melanogenesis through separate pathways. These differences could explain why forskolin is a stronger inducer of *tyrosinase* and *Tyrp1* promoter activities. It should be noted, however, that the effects of forskolin and LY294002 on tyrosinase and Tyrp1 expression and melanin synthesis were roughly comparable, suggesting that

LY294002 could also control melanogenesis through post-transcriptional modifications of tyrosinase or Tyrp1. Post-transcriptional modifications of tyrosinase and Tyrp1 have already been proposed to upregulate their enzyme activity (Imokawa and Mishima, 1982; Martinez-Esparza *et al*, 1998; Park *et al*, 1999). Whether LY294002 could promote such modifications remains to be elucidated.

In conclusion, we demonstrate in this study that the PI3K inhibitor, LY294002, increases Mitf expression, leading to a stimulation of tyrosinase and Tyrp1 expression and finally induction of melanogenesis. Our data suggest that activation of the PKA/CREB cascade and inhibition of PI3K during cAMP-induced melanogenesis cooperate to trigger a stronger induction of melanin synthesis. Taken together, our results bring new information on the molecular mechanisms involved in the cAMP-induced melanogenesis and help in the understanding of the signaling events that govern pigmentation.

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