Glycogen Synthase Kinase 3β Is Activated by cAMP and Plays an Active Role in the Regulation of Melanogenesis*

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In human and mouse, cAMP plays a key role in the control of pigmentation. cAMP, through the activation of protein kinase A, increases the expression of microphthalmia-associated transcription factor (MITF), which in turn stimulates tyrosinase gene expression, to allow melanin synthesis. Beyond this simplified scheme, cAMP inhibits phosphatidylinositol 3-kinase (PI3K), and inhibition of PI3K, by a specific inhibitor, stimulates melanogenesis. However, the link between the PI3K pathway and melanogenesis remained to be elucidated. In this report, we showed that cAMP, through a protein kinase A-independent mechanism, led to inhibition of AKT phosphorylation and activity. Consistent with the role of AKT in the regulation of glycolgen synthase kinase 3β (GSK3β), cAMP decreased the phosphorylation of GSK3β and stimulated its activity. Further, experiments were performed to investigate the role of GSK3β in the regulation of MITF expression and function. We observed that GSK3β regulated neither MITF promoter activity nor the intrinsic transcriptional activity of MITF but synergized with MITF to activate the tyrosinase promoter. Additionally, lithium, a GSK3β inhibitor, impaired the response of the tyrosinase promoter to cAMP, and cAMP increased the binding of MITF to the M-box. Taking into account that GSK3β phosphorylates MITF and increases the ability of MITF to bind its target sequence, our results indicate that activation of GSK3β by cAMP facilitates MITF binding to the tyrosinase promoter, thereby leading to stimulation of melanogenesis.

In mammals, epidermal melanocytes synthesize and transfer melanin pigment to surrounding keratinocytes to allow skin and hair pigmentation. In human, melanins play a crucial protective role against the carcinogenic and deleterious effects of ultraviolet radiation of solar light. Thus, numerous efforts have been made to understand the molecular mechanisms that govern pigment production.

In mice as well as in humans, it is now well established that pro-opiomelanocortin peptides, adrenocorticotropic hormone (ACTH)¹ and α-melanocyte stimulating hormone (αMSH), play a key role in the control of pigmentation. Indeed, any disturbance of αMSH or ACTH signaling, due to mutations of the melanocortin type 1 receptor (MC1R) (1) or to a decrease of αMSH or ACTH expression, results in an inhibition of melanin synthesis (2). Conversely, pathologic overexpression of αMSH (3) or ACTH (4, 5) or administration of αMSH analogue to human voluntaries increase skin pigmentation (6, 7). Binding of ACTH or αMSH to the Gαs-coupled MC1R leads to adenylate cyclase activation, elevation of intracellular cAMP, and activation of the protein kinase A (PKA) (8). The cAMP pathway plays a pivotal role in the regulation of skin pigmentation. Indeed, patients with McCune-Albright syndrome display large hyperpigmented areas caused by an activating mutation in the Gαs protein that controls cAMP level (9, 10). Further, mutations in the type Ia regulatory subunit of PKA, leading to a constitutive activation of PKA, have been described in patients with Carney syndrome characterized by spotty skin pigmentation (11, 12). Finally, the propigmenting effects of αMSH can be mimicked, in vitro, by forskolin that directly binds and activates adenylate cyclase (13, 14). Taken together, these observations clearly demonstrate the meaningful role of the cAMP pathway in the regulation of melanogenesis and skin pigmentation by melanocortins.

CAMP increases melanogenesis mainly through the stimulation of tyrosinase expression, the enzyme catalyzing the rate-limiting reaction of the melanin synthesis process (15–17). We have shown that cAMP, through activation of PKA and CREB transcription factor, promotes an increase in the expression of microphthalmia-associated transcription factor (MITF) (18), a melanocyte-specific transcription factor crucial for melanocyte development and differentiation (19, 20). As a result, MITF binds to and activates the tyrosinase promoter, leading thereby to stimulation of melanogenesis (21–23).

In the course of these studies, we observed that cAMP regulates other signaling pathways that are also involved in the control of melanogenesis. In melanocytes and melanoma cells, elevation of the intracellular cAMP content results in the activation of the Ras/ERK cascade (13). However, activation of Ras/ERK leads to an inhibition of melanogenesis, and this pathway has been thought to be a feedback mechanism preventing an excessive production of melanin that would be toxic for cells (24). Indeed, ERK and RSK (ribosomal S6 kinase), which is activated by ERK, phosphorylate MITF and promote its degradation, thereby leading to an inhibition of tyrosinase expression and melanogenesis (25, 26). Further, in B16 melanoma cells, we showed previously that cAMP inhibits phos-
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Expression Vectors, Transfection, and Luciferase Assays—The luciferase reporter plasmids pTyro and pMITF and the expression vector encoding MITF were described previously (18, 33). The pCDNA, vector encoding GSK3β, was kindly provided by Dr. T. C. Dale (London, UK). B16 melanoma cells were seeded in 24-well dishes, and transient transfections were performed following the protocol using 2 μg of LipofectAMINE and 0.5 μg of total DNA plasmid. pCMV-β-galactosidase 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.

Immunoprecipitation and in Vitro AKT Assay—B16 cells were seeded in 6-well dishes, and transient transfections were performed the following day using 10 μl of LipofectAMINE and 1.5 μg of HA-tagged AKT or 1 μg of HA-tagged AKT with 2 μg of empty pCDNA, or vector encoding RasV12. HA-tagged AKT in the mammalian expression vector pCDNA3 was a kind gift from Dr. G. Baier (Innsbruck, Austria), and the vector encoding RasV12 was provided by Dr. A. Eychene (Orsay, France). Cells were treated for 1 h with 50 μM forskolin or 15 μM LY294002, and then they were lysed in buffer A and immunoprecipitated with the antibody to AKT. AKT was eluted from the affinity matrix using 6 × SDS-sample buffer before adding the labeled probe.

Nuclear Extracts and Gel Mobility Shift Assay—Nuclear extracts from control cells or cells incubated with forskolin for 1 h were prepared as described previously (34). A double-stranded synthetic M-box, 5′-GAAAAAGTCATGTGCTTTGCAGAAGA-3′ from control cells or cells incubated with forskolin for 1 h were prepared as described previously (34). A double-stranded synthetic M-box, 5′-GAAAAAGTCATGTGCTTTGCAGAAGA-3′ was used to produce labeled probe for competition.

RESULTS

In B16 Melanoma Cells, cAMP Leads to an Inhibition of AKT Phosphorylation and Activity—First, we investigated, in B16 melanoma cells, the effects of two cAMP-elevating agents, aMSH and forskolin, on AKT. Immunoblotting of cell lysates with phospho-specific antibodies to AKT revealed that aMSH, a physiologic melanocyte-differentiating agent, as well as forskolin, induced a strong inhibition of AKT phosphorylation on both threonine 308 and serine 473 (Fig. 1A). As expected, LY294002, a specific pharmacological inhibitor of PI3K, also abolished AKT phosphorylation on both residues. Increase in cAMP content led to a complete dephosphorylation of AKT after 30 min (Fig. 1B). The effect of LY294002 was even more rapid since no phosphorylation persisted after 15 min of treatment.

Furthermore, B16 cells, transiently transfected with a vector encoding a HA-tagged AKT were left untreated or exposed to forskolin or to LY294002 for 30 min. Then, HA-AKT was immunoprecipitated from the conditioned media of the transfected cells, and immunoblotting of the immunoprecipitate with phospho-specific AKT revealed that phosphorylation on threonine 308 and serine 473 was reduced to 20% or less of the control value. The specificity of the antibody was confirmed by using a phospho-specific antibody to AKT revealed that phosphorylation on threonine 308 and serine 473 was reduced to 20% or less of the control value. The specificity of the antibody was confirmed by using a phospho-specific

Cell Cultures—B16F10 murine melanoma cells were grown at 37 °C under 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 7% fetal calf serum and penicillin (100 units/ml)/streptomycin (50 μg/ml).

Western Blot Assays—B16 melanoma cells were cultured in 6-well dishes with or without different effector concentrations for the times indicated in the figure legends. Then, cells were lysed in buffer A containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 μg leupeptin, in 1 mM AEBSF, 100 mM NaF, and 10 mM NaVO₄. Supernatants (30 μg) were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then exposed to the appropriate antibodies. Proteins were visualized with the ECL system from Amersham Biosciences using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. Western blot assays were representative of at least three experiments.

Antibodies—The polyclonal phospho-specific AKT (S473 and T308), GSK3β (S9), CREB (S133), p42/p44 MAPK antibodies, and polyclonal antibody that recognizes AKT regardless of its phosphorylation state were from Cell Signaling. The monoclonal GSK3β (0011-A) and monoclonal ERK2 (02-A) antibodies were from Santa Cruz Biotechnology. Monoclonal anti-hemagglutinin (HA) 12CA5 antibody was from Berkeley Antibody. The phospho-glycogen synthase antibody (Ab-1) and the phospho-specific Tau antibody (S396) were purchased from Onco-Regenex. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. Western blot assays were representative of at least three experiments.

One of the key effectors of PI3K is the serine/threonine kinase AKT. In response to growth factors and hormones, AKT is activated by binding to the membrane PI3K phospholipid products and phosphorylation on threonine 308 and serine 473 (28, 29). Activated AKT phosphorylates the glycogen synthase kinase 3β (GSK3β) on serine 9 and promotes its inactivation (30). GSK3β is a serine/threonine kinase, first described to regulate glycogen synthase activity. More recently, GSK3β was demonstrated to be a key mediator of vertebrate development, tumorigenesis, and cell differentiation (31, 32).

Taking into account the role of cAMP in the regulation of melanogenesis, we wished to evaluate the involvement of AKT and GSK3β in the regulation of melanocyte differentiation by cAMP. In this report, we clearly demonstrated, in B16 melanoma cells, that cAMP, by a PKA-independent mechanism, led to inhibition of AKT phosphorylation and activity, resulting in a dephosphorylation and activation of GSK3β. Further, we showed that GSK3β regulated neither MITF transcription nor MITF intrinsic transcriptional activity. However, we found that lithium, a GSK3β inhibitor, decreased the response to cAMP of the tyrosinase promoter. Additionally, we showed that GSK3β synergized with MITF to stimulate the tyrosinase promoter. Finally, short cAMP treatment, which did not up-regulate MITF expression, enhanced the binding of MITF to the M-box sequence of the tyrosinase promoter. Together, our results suggest that activation of GSK3β by cAMP is involved in the regulation of cAMP-induced melanogenesis by up-regulating the binding of MITF to the tyrosinase promoter.

EXPERIMENTAL PROCEDURES

Materials—Forskolin, sodium fluoride, sodium orthovanadate, 4-(2-aminophenoxy)butane-sulfonfonyl fluoride (AEBSF), aprotinin, and clostridium lethal toxin were purchased from Sigma. The PKA inhibitor H89-dihydrochloride and the PI3K inhibitor LY294002 were from Merck Eurolab. Dulbecco’s modified Eagle’s medium, trypsin, and LipofectAMINE reagent were from Invitrogen, and fetal calf serum was from Hyclone. Clostridium sordellii lethal toxin (LT) was a gift from Pro. P. Boquet (Nice, France). The peptide GRPRRTSSFAEG (Crosstide) and the glycogen synthase peptide-2 (GS peptide-2) (5′-GRRAVPPVPSLSRBHSSHQPSDEEEE) were from EuroMeds.

Antibodies—The polyclonal phospho-specific AKT (S473 and T308), GSK3β (S9), CREB (S133), p42/p44 MAPK antibodies, and polyclonal antibody that recognizes AKT regardless of its phosphorylation state were from Cell Signaling. The monoclonal GSK3β (0011-A) and monoclonal ERK2 (02-A) antibodies were from Santa Cruz Biotechnology. Monoclonal anti-hemagglutinin (HA) 12CA5 antibody was from Berkeley Antibody. The phospho-glycogen synthase antibody (Ab-1) and the phospho-specific Tau antibody (S396) were purchased from Oncogene Research products. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were from Dakopatts.

Cell Cultures—B16F10 murine melanoma cells were grown at 37 °C under 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 7% fetal calf serum and penicillin (100 units/ml)/streptomycin (50 μg/ml).

Western Blot Assays—B16 melanoma cells were cultured in 6-well dishes with or without different effector concentrations for the times indicated in the figure legends. Then, cells were lysed in buffer A containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 μg leupeptin, in 1 mM AEBSF, 100 mM NaF, and 10 mM NaVO₄. Supernatants (30 μg) were resolved by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and then exposed to the appropriate antibodies. Proteins were visualized with the ECL system from Amersham Biosciences using horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody. Western blot assays were representative of at least three experiments.
50 and 70%, respectively, indicating that the decrease in AKT phosphorylation correlated with an inhibition of its activity. In each experiment, detection of AKT regardless of its phosphorylation state ensured even loading of each lane (Fig. 1).

To investigate whether cAMP mediates its effect through a PI3K-dependent mechanism, B16 cells were infected with an adenovirus encoding a constitutively active (p110CAAX) or a kinase-dead (p110KD) form of the p110 subunit of PI3K. B16 cells were then exposed or not to forskolin, and phosphorylation of AKT was examined. p110CAAX stimulated the phosphorylation of AKT over the basal and markedly reduced the effect of cAMP as compared with cells infected with a control adenovirus (Fig. 2B). Thus, to define whether cAMP acts independently of Ras to inhibit AKT.

In B16 Cells, cAMP Decreases GSK3β Phosphorylation and Promotes Its Activation—Next, we wanted to determine whether AKT inactivation by cAMP had an impact on downstream events in B16 melanoma cells. To this aim, we focused our attention on GSK3β, a cellular substrate of AKT. Immunoblotting experiments of cells exposed to forskolin or to LY294002 with phospho-specific antibody revealed a decrease of GSK3β phosphorylation on serine 9 as compared with control cells (Fig. 3A). Next, we investigated the effect of this dephosphorylation on GSK3β activity. After treatment of the cells with forskolin or LY294002, GSK3β was immunoprecipitated and subjected to an in vitro kinase assay. Results presented in Fig. 3B show that forskolin and LY294002 stimulated GSK3β activity. Immunodetection of total GSK3β indicated that these differences were not due to variations in the level of protein expression. To confirm the activation of GSK3β, the effects of forskolin and LY294002 on the phosphorylation of endogenous substrates of GSK3β such as the glycogen synthase and the microtubule-associated protein, Tau, were then analyzed in intact cells. Using phospho-specific antibodies, we observed that forskolin and LY294002 increased phosphorylation of both glycogen synthase and Tau protein as compared with cells in basal condition (Fig. 3C). The blot was also incubated with an anti-ERK2 antibody to control for the equal loading of the gel. In conclusion, these results clearly demonstrate that in B16 melanoma cells, GSK3β is activated in response to cAMP.

The Effect of cAMP on AKT and GSK3β Is Not Mediated by PKA and Ras/ERK Pathways—Although PKA is the major cellular target of cAMP, this nucleotide appears to have some PKA-independent actions (35–37). Thus, to define whether PKA was involved in the regulation of AKT and GSK3β follow-
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FIG. 2. cAMP inhibits AKT through a PI3K-dependent, p21ras-independent pathway. B16 cells were infected with an adenovirus encoding either a constitutively active (p110CAAX) or a kinase-dead (p110KD) form of the p110 subunit of PI3K (A). Then, cells were exposed for 1 h to 50 μM forskolin, and AKT phosphorylation was analyzed as described in panel A. B16 cells were transfected with an HA-tagged AKT in the presence of an empty pCDNA3 plasmid or a vector encoding RasV12 and then were incubated or not with 50 μM forskolin for 1 h (B). The activity of AKT was next tested in vitro against Crosstide. Data are expressed as percentage of AKT activity in control cells and are means ± S.E. of three experiments performed in triplicate. Detection of total AKT ensured that each lane was evenly loading.

FIG. 3. cAMP activates GSK3β. B16 melanoma cells were treated as described in the legend for Fig. 1 (panel B), and immunoblotting of cell lysates was done with phospho-specific GSK3β antibody (PS9-GSK3β) (A). Detection of total GSK3β was used to ensure that each lane was evenly loaded. Endogenous GSK3β, from control B16 cells or cells exposed to 50 μM forskolin or 15 μM LY294002 for the indicated time, was immunoprecipitated, and its activity was measured with the GS peptide-2 as substrate (B). The results are expressed as percentage of GSK3β activity in control cells and are means ± S.E. of three experiments performed in triplicate. The level of GSK3β in immunoprecipitate is shown below the graph. Cells were incubated for 1 h with 50 μM forskolin or 15 μM LY294002 and then submitted to immunoblotting using a phospho-specific glycogen synthase (pGS) antibody or an antibody that recognizes Tau phosphorylated on S396 (p-TAU) (C). Detection of ERK2 ensured even loading of each lane. Molecular masses, indicated on the left of Western blots, are expressed in kilodaltons.

Involvement of GSK3β during cAMP treatment, we used H89, a cell-permeable and selective inhibitor of PKA. Treatment of melanoma cells with H89 had no effect on forskolin-induced inhibition of AKT phosphorylation on threonine 308 and serine 473 (Fig. 4A). Immunoblotting also indicated that H89 did not prevent the inhibition of GSK3β phosphorylation on serine 9 induced by forskolin (Fig. 4A). The functionality of H89 was assessed by its ability to block forskolin-induced phosphorylation of CREB at serine 133.
Detection of ERK2 ensured that each lane was evenly loaded. These results clearly indicate that in B16 melanoma cells, PKA is not involved in the regulation of AKT and GSK3β by cAMP.

Interestingly, we have recently reported that cAMP stimulates the Ras/ERK pathway via a PKA-independent mechanism (35). Therefore, we wished to explore the possibility that the Ras/ERK pathway was involved in the effect of cAMP on AKT and GSK3β. To this purpose, we took advantage of C. sordelii LT, which is an inhibitor of p21ras and thus allows the inhibition of the downstream cascade. Immunoblotting with phospho-specific antibodies demonstrated that LT failed to abolish the inhibition of AKT and GSK3β phosphorylation induced by forskolin exposure (Fig. 4B). The functionality of LT was demonstrated by its ability to block forskolin-induced phosphorylation and activation of ERK1/ERK2. Together, these results show that in B16 melanoma cells, cAMP regulates the AKT/GSK3β cascade via a mechanism that involves neither PKA nor Ras/ERK signaling pathways.

Involvement of GSK3β during cAMP-induced melanogenesis—As MITF, through the regulation of tyrosinase expression is a key actor in cAMP-induced melanogenesis, we explored the possibility that GSK3β regulates MITF expression through a stimulation of the transcriptional activity of its promoter. As shown in Fig. 5A, GSK3β did not alter basal or...
cells were transfected with the tyrosinase promoter and then stimulated, the enzyme that controls melanin synthesis. To do so, B16 cells were transfected with the tyrosinase promoter but synergized with MITF to stimulate the tyrosinase transcription. Additionally, we showed that GSK3β activity. Using a mammalian one-hybrid system, we found that GSK3β did not up-regulate the intrinsic transcriptional activity of MITF (Fig. 5B). Finally, we wished to evaluate the potential role of GSK3β in the regulation of tyrosinase expression, the enzyme that controls melanin synthesis. To do so, B16 cells were transfected with the tyrosinase promoter and then exposed to forskolin in presence or absence of lithium, an inhibitor of GSK3β. The results presented on Fig. 6A showed that lithium reduced by 50% the response to cAMP of the tyrosinase promoter. Additionally, we showed that GSK3β by itself did not significantly affect the activity of the tyrosinase promoter but synergized with MITF to stimulate the tyrosinase promoter (Fig. 6B). Together, these data suggest that GSK3β, through the control of MITF function, is involved in the regulation of tyrosinase transcription by cAMP. Finally, gel shift assays were performed with nuclear extracts from control cells or cells that have been exposed to cAMP for 1 h. We observed an increased binding of nuclear proteins from cAMP-treated cells to the M-box probe as compared with control cells (Fig. 6C, lower panel).

The complexes were shifted by specific MITF antibody, indicating that cAMP stimulated the formation of MITF/M-box complexes. Since cAMP did not stimulate MITF expression at 1 h (Fig. 6C, upper panel), we concluded that cAMP increased the ability of MITF to bind its target sequence. Together, these results suggest that GSK3β plays a key role in cAMP-induced melanogenesis by enhancing the binding of MITF to the tyrosinase promoter.

**DISCUSSION**

In the course of investigating the molecular mechanisms involved in the regulation of melanocyte differentiation, we have observed previously, in B16 cells, an inhibition of PI3K during cAMP-induced melanogenesis (27). Since the inhibition of PI3K stimulates melanogenesis, we wished to dissect, in the present report, the molecular events that connected the PI3K pathway to melanin synthesis. We first focused our attention on AKT, a well characterized kinase that functions downstream of PI3K. Depending on the cell type, cAMP has been reported to inhibit or stimulate AKT (38, 39). Thus, we verified the effect of cAMP on AKT in B16 melanoma cells. Our results showed that cAMP potently inhibited the phosphorylation of AKT at threonine 308 and serine 473 and led to an inhibition of AKT activity. Additionally, the effect of cAMP on AKT was abolished in the presence of a constitutively active mutant of PI3K, demonstrating that cAMP promotes an inhibition of AKT evoked by cAMP, indicating that the inhibition of AKT by cAMP is mediated through a p21ras-independent pathway.
pathway. It should be noted that we have reported previously an activation of p21ras by cAMP in melanocytes and melanoma cells (35). Thus, in the conditions in which we observed an inhibition of Akt, p21ras is activated, demonstrating that the activation of p21ras cannot prevent the inhibition of Akt.

Akt phosphorylates GSK3β at serine 9, leading to GSK3β inactivation. Therefore, the inhibition of Akt by cAMP should lead to the activation of GSK3β. However, it has also been reported that PKA directly phosphorylates GSK3β at serine 9 and inhibits its activity (39, 40). In B16 cells, cAMP decreased the phosphorylation of GSK3β at serine 9 and led to the stimulation of its kinase activity. Consistent with the effect of Akt on GSK3β and the effect of cAMP on Akt, we demonstrate for the first time that cAMP promotes an activation of GSK3β.

As PKA is the major intracellular target of cAMP, it was tempting to propose that the effect of cAMP on Akt and GSK3β involved the PKA. However, a pharmacological inhibitor of PKA did not impair the effect of cAMP on Akt and GSK3β. As mentioned above, in B16 cells, cAMP also stimulates the Ras/ERK pathway via a PKA-independent mechanism (35). Thus, we hypothesized that the activation of the Ras/ERK pathway could mediate the effect of cAMP on Akt and GSK3β. However, inhibition of p21ras, and consequently of ERK, by C. sordellii lethal toxin did not alter the effect of cAMP on Akt and GSK3β. Thus, cAMP leads to an inhibition of Akt and a stimulation of GSK3β through PKA and p21ras/ERK-independent pathways. Recently, it has been also reported that cAMP inhibits Akt through a mechanism that does not involve PKA (41).

GSK3β has been widely implicated in cell homeostasis by its ability to phosphorylate a broad range of substrates including the glycogen synthase, the microtubule-associated protein Tau, and β-catelin (42). Interestingly, cAMP-induced activation of GSK3β stimulated phosphorylation of the microtubule-associated protein, Tau (Fig. 2C). Considering the role of Tau in the organization of microtubule and actin cytoskeleton (43, 44), as well as in the regulation of organelle traffic (45), our results point to a potential role of GSK3β and Tau in the regulation of cAMP-induced dendritogenesis and melanosome transport, two parameters of melanocyte differentiation.

Further, phosphorylation of β-catelin by GSK3β promotes its degradation and prevents its translocation to the nucleus, where β-catelin acts in concert with the lymphoid enhancer factor-1/T-cell factor (LEF-1/TCF) family of transcription factors to transactivate target genes. The promoter of MITF contains a LEF-1 binding site, and β-catelin stimulates the transcriptional activity of the MITF promoter (46). Thus, the activation of GSK3β following cAMP exposure should lead to an inhibition of the MITF promoter activity due to β-catelin degradation. However, in B16 cells, elevation of the cAMP content did not induce the phosphorylation of β-catenin and did not affect the activity of a TOPFlash reporter plasmid bearing LEF-1 binding sites (data not shown). Cotransfection of MITF promoter reporter construct with GSK3β confirms that GSK3β did not regulate the transcriptional activity of the MITF promoter (Fig. 5A).

Recently, Takeda et al. (47) have demonstrated that GSK3β phosphorylates MITF on serine 298, thereby enhancing its binding to the tyrosinase promoter. Interestingly, mutation of the MITF serine 298 has been identified in patients with Waardenburg syndrome type II, which is characterized by pigmentedary disorders, emphasizing the physiopathologic importance to understand the role of GSK3β in the control of pigmentation. Therefore, since GSK3β stimulates neither the MITF promoter activity nor the intrinsic transcriptional activity of MITF, we hypothesized that cAMP, through GSK3β activation, could increase the ability of MITF to bind its target sequence. Evidence provided by the work taken from Takeda et al. (47), we demonstrated that GSK3β inhibited the action of MITF on the tyrosinase promoter. Additionally, lithium, a GSK3β inhibitor, decreased cAMP-stimulated induction of the tyrosinase promoter. Finally, we demonstrated that cAMP increases the binding of MITF to the M-box sequence. To demonstrate the key role of the serine 298 phosphorylation, we studied the effect of an MITF mutant, MITF S298A. This mutant cannot be phosphorylated by GSK3β, and thus the effect of this mutant should not be increased by GSK3β or by cAMP. However, in agreement with the previous observation of Takeda et al. (47), this mutant is devoid of any transcriptional activity (data not shown). Further, we constructed two other mutants in which the serine 298 was replaced either by an aspartate or by a glutamate. The positive charge of these amino acids was expected to mimic the charge brought by the phosphate group. Unexpectedly, these two mutants were also unable to transactivate the tyrosinase promoter (data not shown).

Together, these results show that in parallel to the PKA/CREB pathway, which stimulates MITF expression, cAMP, through the activation of GSK3β, increases the ability of MITF to bind to the tyrosinase promoter. These two pathways cooperate, allowing cAMP-elevating agents to efficiently stimulate tyrosinase transcription and thereby melanogenesis. In conclusion, elevation of intracellular cAMP leads to the activation of a complex network of signaling pathways that converge to MITF to control melanin synthesis and melanocyte differentiation (Fig. 7). Our findings, demonstrating that GSK3β plays an active role in cAMP-induced melanogenesis and in melanocyte differentiation, bring information of paramount importance on the molecular mechanisms that control melanin synthesis and skin pigmentation.

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