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Role Played by Microphthalmia Transcription Factor Phosphorylation and Its Zip Domain in Its Transcriptional Inhibition by PIAS3

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Mutation of microphthalmia transcription factor (MITF) results in deafness, bone loss, small eyes, and poorly pigmented eyes and skin. A search for MITF-associated proteins, using a mast cell library that was screened with a construct that encodes the basic helix-loop-helix leucine zipper (Zip) domain of MITF, resulted in the isolation of the STAT3 inhibitor, PIAS3. PIAS3 functions in vivo as a key molecule in suppressing the transcriptional activity of MITF. Here, we report that the Zip domain is the region of MITF that is involved in the direct interaction between MITF and PIAS3. Additionally, we investigated the effect of phosphorylation of MITF on its interaction with PIAS3. We found that phosphorylation of MITF on serines in positions 73 and 409 plays an important role in its association with PIAS3. This effect was profound with phosphorylation on Ser409, which significantly reduced the inhibitory effect of PIAS3 on MITF and also modulated the transcriptional activity of MITF. Thus, phosphorylation of MITF could be considered a fine, and alternative, tuning of its transcriptional machinery.

The microphthalmia transcription factor (MITF) is a basic helix-loop-helix leucine zipper (bHLH-Zip) DNA-binding protein (10). Its gene resides at the *mi* locus in mice (12), and mutation of this gene (21) results in deafness, bone loss, small eyes, and poorly pigmented eyes and skin. The primary cell types affected in MITF-deficient mice are mast cells, osteoclasts, and melanocytes (21). Furthermore, its expression in melanocytes was recently used as a sensitive and specific marker for malignant melanoma (5). In humans, mutation in this gene causes Waardenburg syndrome type II (34). MITF regulates the expression of mouse mast cell protease 6 (mMCP-6) (25), mMCP-5 (22), c-kit (13), p75 nerve growth factor (22), granzyme B (15), and tryptophan hydroxylase (14). MITF also regulates the transcription of the genes that encode tyrosinase (albino locus), tyrosinase-related protein, and pink-eyed Pmel 17 (silver) (2), and recently it was observed that it is also involved in the regulation of Bcl-2 expression (19).

MITF regulates gene transcription by binding to E-box-type enhancers in the 5′ flanking regions of MITF-responsive genes (25). Like many other DNA-binding proteins, the transcription-enhancing activity of MITF is influenced in a complex manner by an array of different intracellular proteins. For example, in vitro studies have indicated that MITF can form heterodimers with the four related family members TFEB, TFEC, TFE3, and USF2 (1, 3, 26, 41).

Other MITF-interacting proteins, PKCI/Hint (31) and PIAS3 (18), were previously identified using the yeast two-hybrid system with the bHLH-Zip domain as bait. These two MITF-associated proteins were shown to be repressors of MITF transcriptional activity (18, 31).

PIAS3 was initially found through its interaction with STAT3, whose transcriptional activity it inhibits (4). In vitro DNA-binding analysis suggested that PIAS3 can block the DNA-binding activity of STAT3 and thus inhibit STAT3-mediated gene activation (4). As mentioned above, the function of PIAS3 as a repressor of MITF-induced transcriptional activity has been demonstrated by inhibiting its DNA-binding activity, and it has been shown that STAT3 does not interfere either in vitro or in vivo with the interaction between PIAS3 and MITF (18). In order to further characterize the interaction between PIAS3 and MITF, we have determined the region within the bHLH-Zip domain that contains the site of interaction.

Phosphorylation-dephosphorylation of proteins is a key step in the regulation of protein-protein interactions. For instance, p300/CBP is an MITF transcriptional coactivator which can associate with MITF only in its MAPK-mediated phosphorylated form (29). Another example is the fact that tyrosine phosphorylation of STAT3 is a prerequisite for its interaction with other STAT proteins that allow the translocation of the STAT dimer into the nucleus, where it activates specific genes (32). Moreover, PIAS3 can heterodimerize with phosphorylated STAT3 only in order to block its DNA-binding activity (4). Therefore, it is likely that MITF participates in the coordinated expression of multiple genes by association-dissociation from proteins as a result of protein kinase-dependent phosphorylation.

Three serine sites for MITF phosphorylation have been reported (8, 35, 40). Phosphorylation of Ser73 (8) and Ser409 (40) occur as a result of Kit ligand stimulation and activation of MAPK and RSK-1, respectively. These serine sites are of interest, since upon their phosphorylation, MITF transcriptional activity is up regulated, and this phosphorylation also serves as a signal for the degradation of MITF by ubiquitin-dependent proteolysis in melanocytes (40). Ser298 is phosphorylated in vitro by GSK3β, although it has the consensus sequence to be a MAPK phosphorylation site in vivo (35). Ser298 is one of the known mutation sites in Waardenburg syndrome type II (36).

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This site is located close to the bHLH-Zip domain, and its phosphorylation increases MITF binding activity in vitro (35).

The effect of MITF phosphorylation on its interaction with other proteins was further investigated in the present work. We determined how phosphorylation of three serine sites on MITF, 73, 298, and 409, regulates MITF transcriptional activity via its association with PIAS3. Phosphorylation of Ser409 was found to significantly decrease the association of MITF with PIAS3 and thus reconstituted its transcriptional activity. Moreover, in this work we demonstrate that the Zip domain of MITF contains the site of its interaction with PIAS3.

MATERIALS AND METHODS

Cell culture and treatments. BL6-B16 melanoma cells were cultured in a growth medium containing RPMI 1640, 10% fetal calf serum, 2 mM L-glutamine, 2 mM nonessential amino acids, 100 U of penicillin/ml, 100 μg of streptomycin (Gibco Invitrogen Corp.)/ml, and 50 μM β-mercaptoethanol (Fisher Scientific, Medford, Mass.). NIH 3T3 cells were cultured and maintained in the same medium. Femoral bone marrow cells derived from $MITF^{sp/sp}$ and $MITF^{di/di}$ mice were cultured in IL-3-containing medium for 3 weeks to generate bone marrow-derived mast cells (BMMC) as previously described (30). All of the cells were grown in a humidified incubator at 37°C with 5% CO_2.

Mice. Mouse colonies were established from transgenic mice ($MITF^{sp/sp}$ and $MITF^{sp/di}$) kindly provided by Lynne Lamoreux from the College of Veterinary Medicine, Texas A&M University. The MITF encoded by the mutant mi^{di} allele lacks the Zip domain (7) due to a C-to-T transition in exon 8 at position 916, which introduces a premature stop codon between the bHLH and the leucine zipper domain.

Plasmid construction. Mouse MITF (1,129 bp) was subcloned into the *Xba*I and *Hin*dIII sites of the pcDNA3.1 (−) vector (Invitrogen). This vector was used for the production of all the MITF mutants by site-directed mutagenesis (Invitrogen), in which serine was replaced by aspartate at position 73, 298, or 409 (S73D, S298D, and S409D, respectively). Double mutation at serines 73 and 409 produced the S73/409D mutant. The cDNA encoding the open reading frame of mouse PIAS3 was subcloned into the *Xba*I and *Hin*dIII sites of the pcDNA3.1 (−) vector. Mouse MITF (1,129 bp) was inserted into the pGEX-4T-3 vector (Stratagene). The fidelity of all constructs was verified by direct sequencing. The luciferase reporter plasmid pSP72, containing the MITF binding region of the promoter and the first exon of the mMCP-6 gene (−191 to + 26), as well as a construct with a deleted MITF binding site (−151 to + 26), were generously provided by Y. Kitamura, Osaka, Japan. The pEBB-Mi (S409A) plasmid was kindly provided by David E. Fisher, Boston, Mass. A plasmid containing the constitutively active form of RSK (pk3h.rsk.y707a) was kindly provided by Tom Sturgill, Charlottesville, Va. (28).

Transient cotransfection and luciferase assay. NIH 3T3 cells (5×10^5) were cotransfected with liposomes (Promega) with 0.1 μg of the luciferase reporter, 0.1 μg of pcDNA-MITF, 0.1 μg of pcDNA-PIAS3, or 0.1 μg of pcDNA alone as a nonspecific control. The cells were incubated in 24-well plates for 48 h, lysed, and assayed for luciferase activity. The luciferase activity was normalized to the total protein concentration. The normalized value was then divided by the luciferase activity obtained by cotransfection of the reporter with pcDNA alone. The ratio was expressed as the relative luciferase activity.

Primers. The bacteriophage T7 transcription regulatory element and ribosome binding site were fused with the MITF primers. The pcDNA3.1 mouse MITF was used as a template. The basic-domain primers were as follows: sense, 5′-C TAA TAC GAC TCA CTA TAG GGA AGG AGA TAT ACA T ATG ATG CAG GGC CTG CCA CCG CCA GGC; antisense, 5′-TTA TCT TCT TCT TCG TTC AAT CAA GTT. The HLH domain primers used were as follows: sense, 5′-C TAA TAC GAC TCA CTA TAG GGA AGG AGA TAT ACA T ATG ATG TTT AAC ATA AAC GAC CGC ATT; antisense, 5′-TTA TTC CCG TTG CAA CTT CCG GAT GTA. The primers for the leucine zipper domain were as follows: sense, 5′-C TAA TAC GAC TCA CTA TAG GGA AGG AGA TAT ACA T ATG ATG CAG CAA CGA GCT AAG GAC CTT; antisense, 5′-TTA CAG CTC CTG TAC TCT GAG CAG CAG. The primers for the downstream leucine zipper domain were as follows: sense, 5′-C TAA TAC GAC TCA CTA TAG GGA AGG AGA TAT ACA T ATG ATG GAG ATG CAG GCT AGA GCG CA; antisense, 5′-TTA AAG TTC CTG GCT GCA GTT CTC AAG.

In vitro GST pull-down assay. The glutathione *S*-transferase (GST)-PIAS3 fusion protein was expressed in protease-deficient *Escherichia coli* strain B12 and purified on glutathione-Sepharose beads (Amersham Biosciences) essentially as described before (18). Pull-down assays (6) were performed with the GST-PIAS3 fusion protein (1 to 5 μg) bound to Sepharose beads and preincubated for 1 h at 4°C in 1 ml of binding buffer (phosphate-buffered saline [PBS], 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.1% NP-40). One to 10 μl of ^{35}S-labeled full-length MITF (wild type or mutant) or its fragments were synthesized using the TNT-coupled rabbit reticulocyte lysate system (Promega) and then added to each preincubation mixture, and the binding reaction was carried out overnight at 4°C. Beads were washed four times in 1 ml PBS- 290 mM NaCl and boiled for 7 min in sample buffer, and aliquots were examined by electrophoresis. The integrity and quantity of GST fusions were confirmed by Blue stain reagent (Pierce), and autoradiography detected the amount of retained full-length MITF and its fragments.

Coimmunoprecipitation. BMMC derived from $MITF^{di/di}$ and $MITF^{sp/sp}$ mice, BL6-B16 melanocyte cells, and NIH 3T3 cells were used in various coimmunoprecipitation experiments. BL6-B16 cells were cotransfected with MITF and PIAS3. NIH 3T3 cells were cotransfected with constitutively active RSK, PIAS3, and MITF, wild type or mutant. BL6-B16 cells were exposed to either 100 nM tetradecanoyl phorbol acetate (TPA) or 80 μg of c-kit ligand/ml (31) for up to 30 min (40). The cells (5×10^6 to 10×10^6) were lysed by the addition of 300 μl of cold lysis buffer (0.01 M Tris-HCl, pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, and 0.25 μM phenylmethylsulfonyl fluoride) and 15 μl of protease inhibitors (Sigma). The cells were then homogenized, and their supernatants were collected after a 15-min centrifugation in a microcentrifuge at 4°C. The recovered lysates were incubated with either anti-mouse MITF antibody (31), MITF monoclonal antibody C5 (38), or anti-mouse PIAS3 antibody (Santa Cruz), prebound to 15 mg of protein A/G-agarose (Pierce), and incubated with agitation overnight at 4°C. Recovered immune complexes were washed three times with lysis buffer. All buffers contained protease inhibitors.

Gel electrophoresis and Western blotting. Proteins were resolved by SDS-10% polyacrylamide gel electrophoresis (PAGE) under reducing conditions and transferred to 0.45-μm-pore-size nitrocellulose membranes. The blots were probed with either anti-PIAS3, anti-MITF, or anti STAT3 (Santa Cruz) antibody. Visualization of reactive proteins was done by enhanced chemiluminescence (18).

Indirect fluorescent immunocytochemistry. c-kit ligand (50 μg/ml)-activated BL6-B16 cells were transfected with green fluorescent protein (GFP)-PIAS3 alone, whereas NIH 3T3 cells were cotransfected with GFP-PIAS3 and MITF, either wild type or mutant. The cells were grown on glass coverslips in six-well plates. After being extensively washed with PBS, the cells were fixed with 1.5 ml of 3.7% formaldehyde in PBS for 10 min. The fixed cells were then washed with PBS and permeabilized with 1.5 ml of Triton X-100 diluted 1:2 with PBS containing 7.5 mg of bovine serum albumin. After being blocked for 45 min with normal donkey serum, the cells were stained with rabbit anti-MITF (26) followed by the addition of Red-Cy5-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch). Fluorescence analysis was performed using the Zeiss LSM 410 confocal laser scanning system connected to a Zeiss Axiovert 135 M microscope. The green fluorescence of GFP-labeled PIAS3 was excited with an argon laser (488-nm excitation line with 515-nm long pass barrier filter). Red-Cy5-conjugated goat anti-rabbit antibody was simultaneously excited with an He-Ne laser (543-nm excitation line with 570-nm long pass barrier filter).

RESULTS

The Zip domain of MITF binds directly to its transcriptional inhibitor, PIAS3. In a previous study, the direct association between MITF and PIAS3 was determined using an in vitro pull-down assay (18). The same system was used in this study to identify the region of MITF that is responsible for this interaction based on the region that was used as bait for PIAS3 in a yeast two-hybrid experiment (18) (Fig. 1A). PCR fragments coding for the various domains of MITF were used for the assay. PIAS3 was expressed in bacteria as a GST fusion protein, immobilized on glutathione-Sepharose beads, and assayed for its ability to retain the in vitro-translated MITF domains labeled with [^{35}S]methionine.

The results clearly show that the Zip domain is responsible for the direct interaction with PIAS3 (Fig. 1B). The bHLH domain, the basic domain (594 to 641 bp; data not shown), and

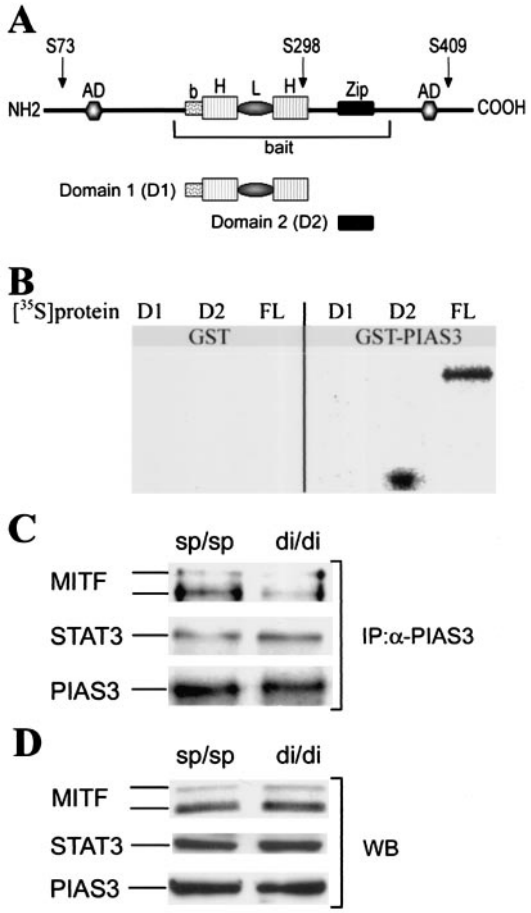

FIG. 1. PIAS3 binds to the MITF leucine Zip domain. (A) Schematic representation of the full-length mouse MITF, including the bHLH-Zip of MITF that was used as bait in yeast two-hybrid screening, showing the structures of the two domains of MITF investigated in the present work, bHLH and Zip. (B) Fragments, bHLH (D1) and Zip (D2), and full-length MITF (FL) labeled with [^{35}S]methionine in vitro and added to GST-PIAS3 immobilized on glutathione-Sepharose beads. Retained ^{35}S-labeled full-length MITF or its fragments were determined by SDS-PAGE and autoradiography. One representative of three experiments is shown. (C) Coimmunoprecipitation (IP) of PIAS3 with MITF or STAT3 in BMMC derived from MITF$^{di/di}$ mice (mutant animals with a Zip domain deletion in MITF in a MITF$^{sp/sp}$ genetic background) or MITF$^{sp/sp}$. The coimmunoprecipitation was performed with anti-PIAS3 (α-PIAS3), and the blot was probed with either anti-MITF or anti-STAT3 antibody, stripped, and reprobed with anti-PIAS3 antibody. One representative of three experiments is shown. (D) Western blot (WB) analysis of BMMC lysates derived from MITF$^{sp/sp}$ or MITF$^{di/di}$ mice. One representative of two experiments is shown.

the HLH region (642 to 779 bp; data not shown) did not bind to PIAS3. In order to further investigate the importance of the Zip domain for this interaction, we determined the in vivo association between PIAS3 and MITF isolated from BMMC derived from MITF$^{di/di}$ mice, whose MITF lacks this domain (7), using a coimmunoprecipitation assay. Control mast cells were derived from their MITF$^{sp/sp}$ littermates, whose MITF has an intact Zip domain. BMMC lysates were incubated with anti-PIAS3 antibody (Fig. 1C) prebound to protein A/G. Blots were probed with either anti-MITF or anti-STAT3 antibody and then stripped and reprobed with anti-PIAS3 antibody to show the amount of PIAS3 bound to the beads. A significant decrease in the association between PIAS3 and MITF was observed in BMMC derived from MITF$^{di/di}$ mice. In contrast, a slight increase was seen in the association between PIAS3 and STAT3 in BMMC derived from MITF$^{di/di}$ mice (Fig. 1C). Similar results were obtained when anti-MITF antibody was prebound to protein A/G (data not shown).

In order to determine the relative amounts of these three proteins in BMMC derived from MITF$^{di/di}$ mice compared to BMMC derived from MITF$^{sp/sp}$ mice, Western blot analysis was performed. The blots were probed with anti-MITF, anti-PIAS3, or anti-STAT3 antibody. No significant change in the cellular concentrations of these proteins was observed in cells derived from the mutated mice (Fig. 1D).

Thus, the results obtained from the pull-down assay, together with those obtained from cells derived from the MITF-truncated mice, clearly indicate that the MITF Zip domain plays an essential role in the interaction between MITF and PIAS3.

Dynamic association of MITF and PIAS3 in activated cells. The subcellular localization of MITF and PIAS3 in c-kit ligand-activated BL6-B16 cells was determined using confocal laser scanning analysis. The cells were transfected with PIAS3 that was constructed with GFP (GFP-PIAS3) or with GFP alone. Resting and activated cells were fixed and stained with rabbit anti-MITF antibody, which was detected by staining with Red-Cy5-conjugated goat anti-rabbit antibody (Fig. 2A). MITF and PIAS3 were located in both the nuclei and the cytosol of resting (Fig. 2A, I) and activated (Fig. 2A, II and III) cells; however, a clear increase in the nuclear colocalization of these two proteins in the transfected cells was seen after the melanocytes were activated by c-kit ligand for 10 min (Fig. 2A, II). Thirty minutes after activation, a decrease in this colocalization was observed (Fig. 2A, III). Interaction between the two proteins was also observed in the nuclei of the resting cells, but to a much lesser extent. Thus, activation of melanocytes for 10 min induced an increase in the association between PIAS3 and MITF, which was reduced by 30 min of activation.

In order to investigate further this dynamic association between MITF and PIAS3, two coimmunoprecipitation experiments were carried out (Fig. 2B). BL6-B16 cells overexpressing MITF and PIAS3 were produced by cotransfection with the appropriate plasmids. The cells were then activated by TPA or c-kit ligand, and the cells were lysed at different times. The BL6-B16 cell lysates were incubated with anti-PIAS3 antibody prebound to protein A/G. The recovered and resolved immune complexes showed the coimmunoprecipitation of PIAS3 with MITF (Fig. 2B). Thirty minutes after activation, a decrease in MITF-PIAS3 association was observed compared to that after 10 min.

The amounts of PIAS3 bound to the beads were similar at all time points (Fig. 2B). Western blots of the same cell lysates were probed with anti-MITF antibody. Similar amounts of MITF were observed 10 and 30 min after TPA activation (Fig. 2B).

A similar pattern of disassociation was seen after 10 min of activation with c-kit ligand (data not shown).

Involvement of MITF-dependent RSK phosphorylation in its interaction with PIAS3. It was previously reported that

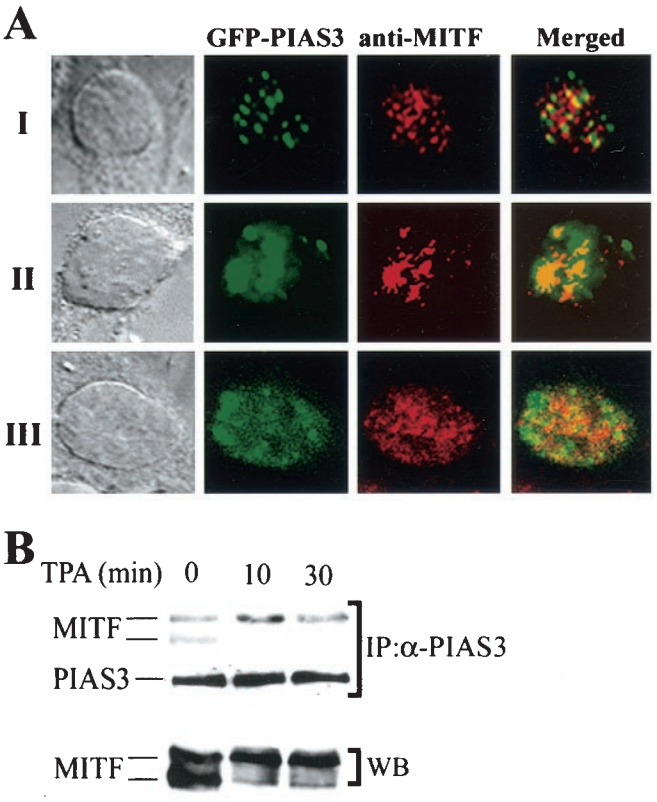

FIG. 2. Nuclear colocalization of MITF and PIAS3 in activated melanocytes (BL6-B16 cells). (A) BL6-B16 melanocyte cells were transfected with GFP-PIAS3 construct (green staining) and immunostained with anti-MITF antibodies and a Cy5-labeled secondary antibody (red staining). The yellow signal indicates colocalization of PIAS3 and MITF in the nuclei. The cells were nonactivated (I) or activated by c-kit ligand for 10 (II) or 30 (III) min and analyzed by laser confocal microscopy. General cell structure is shown in the left-hand (black and white) images. (B) Kinetics of MITF and PIAS3 association in TPA-activated BL6-B16 cells. Coimmunoprecipitation (IP) of PIAS3 with MITF at defined time points in TPA-activated BL6-B16 cells. The cells were transfected with pcDNA-MITF and pcDNA-PIAS3. The blots were probed with anti-MITF antibodies and then reprobed with anti-PIAS3 antibody. Lysates from cell extracts were probed with anti-MITF antibody. One representative of three experiments is shown. WB, Western blot.

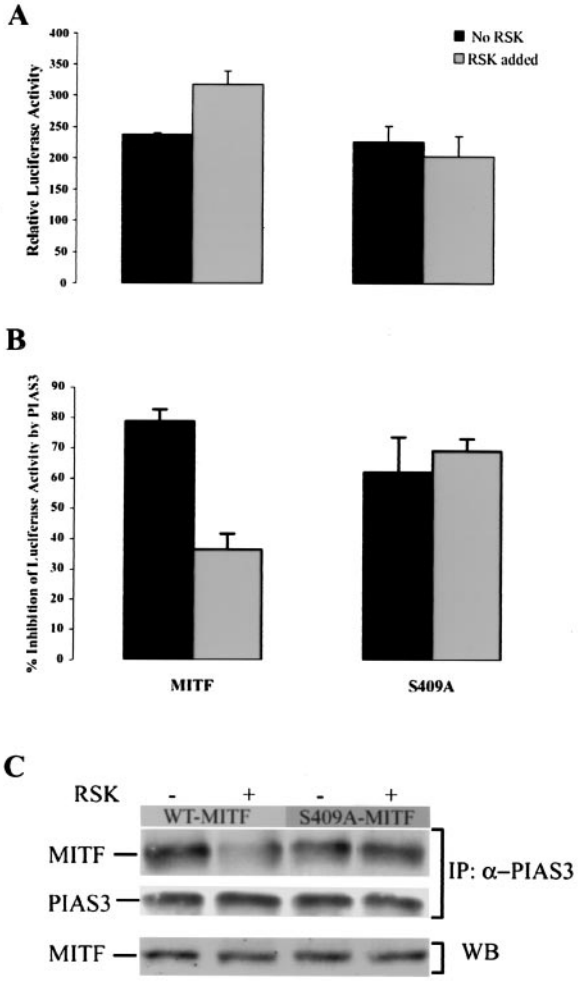

FIG. 3. RSK decreases the association of PIAS3 with MITF and its inhibitory effect on MITF transcriptional activity. (A) NIH 3T3 cells were cotransfected with MITF or its S409A mutant, with a luciferase reporter under the control of the mMCP-6 promoter and with a constitutively active RSK plasmid. (B) Percent inhibition of MITF transcriptional activity by PIAS3. A luciferase reporter plasmid containing a promoter region of mMCP6 was cotransfected to NIH 3T3 cells with either MITF or the S409A MITF mutant, PIAS3, and a constitutively active RSK plasmid. The relative MITF transcriptional activity was determined as for panel A. The results presented in panels A and B were normalized to the total protein amount, and four experiments are shown (means plus standard errors). (C) Coimmunoprecipitation (IP) from NIH 3T3 cells cotransfected with PIAS3 and MITF or its S409A mutant in the presence (+) or absence (−) of constitutively active RSK plasmid. The blots were probed with anti-MITF antibodies. Stripping was performed, and the membrane was reprobed with anti-PIAS3 (α-PIAS3) antibody as a control. Lysates from cell extracts were probed with anti-MITF antibody. WB, Western blot.

MITF is phosphorylated by RSK at serine 409 (23). In order to evaluate the role played by RSK in MITF-PIAS3 interactions, NIH 3T3 cells were cotransfected with a constitutively active RSK plasmid (28), with a luciferase reporter plasmid containing the mMCP-6 promoter, and with either MITF or an S409A MITF mutant which cannot be phosphorylated by RSK. The transcriptional activity of MITF was determined as relative luciferase activity. No significant change in such activity was detected regardless of whether the cells were transfected with MITF or its mutated form with or without RSK (Fig. 3A).

The role played by RSK in the suppression of MITF transcriptional activity by PIAS3 was then determined. The inhibition of MITF transcriptional activity by PIAS3 was determined by a relative luciferase assay (Fig. 3B). The transfection of constitutively active RSK caused a significant decrease in the ability of PIAS3 to inhibit MITF (36% ± 5%; mean ± standard error) compared to cells that were not transfected with RSK (78% ± 4%; mean ± standard error). This decrease was not observed in cells that were transfected with the S409A mutant. Coimmunoprecipitation of PIAS3 and MITF was carried out using the same NIH 3T3 lysates. A significant decrease in the association between PIAS3 and MITF was observed only in cells that were transfected with both wild-type MITF and RSK (Fig. 3C). However, active RSK did not cause any change

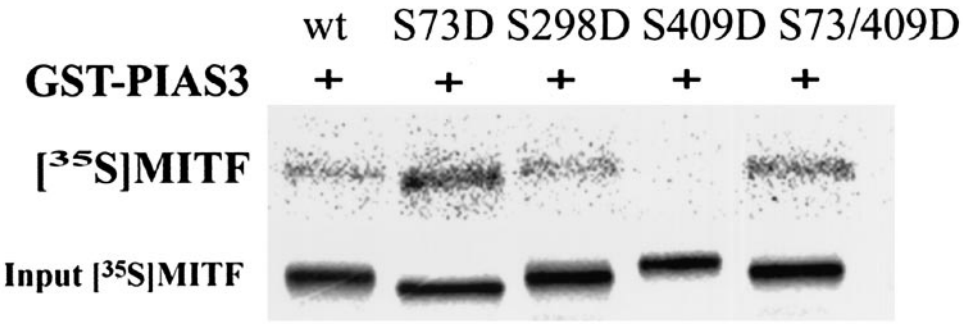

FIG. 4. Direct in vitro interaction between MITF phosphorylated forms and PIAS3. ^{35}S-labeled wild-type (wt) MITF or its mutants with aspartate substituted (S73D, S298D, S409D, and S73/409D) were incubated with (+) GST-PIAS3 immobilized on glutathione-Sepharose beads. Retained ^{35}S-labeled MITF proteins were determined by SDS-PAGE and autoradiography. The amount of in vitro-translated (input) MITF is shown as a control. One representative of three experiments is shown.

in the association between MITF and PIAS3 in cells that contained the S409A MITF mutant. Stripping the membrane and reprobing with anti-PIAS3 antibody revealed a similar amount of PIAS3 bound to the beads. The same amount of MITF was detected in each of the transfected cells, as revealed by probing the lysates with anti-MITF antibody as a control.

Phosphorylation of MITF at S409 interferes with PIAS3 interaction. We chose to evaluate the serine sites in MITF, the biological effects of whose phosphorylation have already been studied in other systems (8, 40, 35). These serine sites are localized at position 73, which is 40 amino acids upstream of the N terminus activation domain; at position 409, which is 40 amino acids downstream of the C terminus activation domain; and at position 298, which is located 9 amino acids after the termination of the Zip domain (33). Site-directed mutagenesis was performed for these three well-characterized MITF phosphorylation sites by replacing the serine with aspartate. Such an amino acid substitution of aspartate for serine can be used to mimic the charge brought by the phosphate group (11, 39).

PIAS3 was expressed in bacteria as a GST fusion protein, immobilized on glutathione-Sepharose beads, and assayed by pull-down assay for its ability to retain in vitro-translated wild-type MITF and its mutants (S73D, S298D, S409D, and S73/409D), which were labeled with [^{35}S]methionine (Fig. 4). More MITF mutant S73D than wild-type MITF or mutant S298D bound to PIAS3. No interaction was observed between PIAS3 and the MITF mutant S409D, whereas double-mutated MITF, S73/409D, showed a slight decrease in binding to PIAS3 compared to S73D. Similar amounts of the in vitro-translated MITF were seen in the various forms of MITF. The GST pull-down alone did not interact with either wild-type MITF or its mutants (data not shown).

S409D mutation of MITF inhibits its nuclear association with PIAS3. Interaction in vivo between PIAS3 and the various mutated forms of MITF was first analyzed using confocal laser scanning microscopy in NIH 3T3 cells cotransfected with GFP-PIAS3 and wild-type MITF or its mutants (Fig. 5A). Almost no nuclear colocalization was observed between PIAS3 and the MITF mutant at S409D; however, mutation at S73D increased the colocalization of the two proteins compared to the S409D mutant.

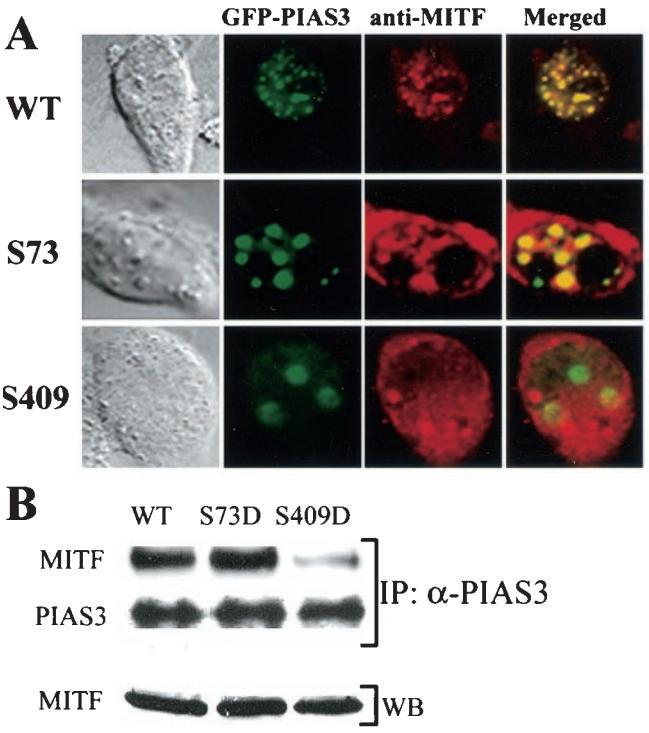

FIG. 5. Nuclear colocalization of PIAS3 and the phosphorylated mutants of MITF in NIH 3T3 cells. (A) NIH 3T3 cells were cotransfected with the GFP-PIAS3 construct and with wild-type (WT) MITF or with the various MITF mutants (S73D and S409D) and with GFP-PIAS3. The immunostaining was performed with anti-MITF antibodies using a Cy5-labeled secondary antibody (red) and was analyzed by laser confocal microscopy. Cell structure is shown in the left-hand (black and white) images. The nuclear colocalization is shown in the right-hand panels (yellow areas). One representative experiment of three is shown. (B) Coimmunoprecipitation (IP) of PIAS3 with MITF or its mutant forms (S73D and S409D) from transfected NIH 3T3 cells using anti-PIAS3 (α-PIAS3) antibody bound to protein A/G. The blots were probed with anti-MITF antibody. Stripping was performed, and the membrane was reprobed with anti-PIAS3 antibody as a control. The lysates were also probed with anti-MITF antibody as a control. One representative experiment of three is shown. WB, Western blot.

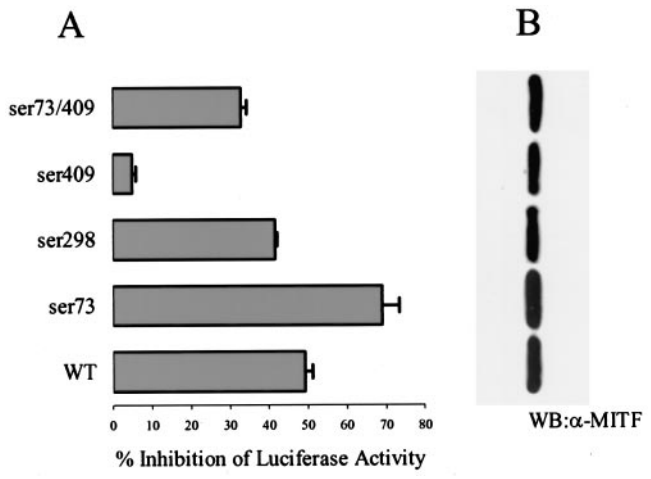

FIG. 6. Inhibition of transcriptional activity of S/D mutants of MITF by PIAS3. (A) Luciferase reporter plasmid, containing a promoter region of mMCP-6, was cotransfected with either wild-type (WT) MITF or each of the MITF mutants (S73D, S298D, S408D, or S73/409D) and PIAS3. The relative MITF transcriptional activity was determined as for Fig. 3. One representative experiment of three is shown. The error bars indicate standard errors. (B) Western blot (WB) analysis was performed on cell lysates of NIH 3T3 cells transfected with the S/D mutants or wild-type MITF. The blots were probed with anti-MITF antibody (α-MITF). One representative of two experiments is shown.

Coimmunoprecipitation was carried out next by incubating transfected NIH 3T3 lysate with anti-PIAS3 antibody prebound to protein A/G. The recovered and resolved immune complexes showed the coimmunoprecipitation of PIAS3 with MITF (Fig. 5B). A significant decrease in association between PIAS3 and MITF was observed only with the S409D mutant, whereas the double mutant S73/409D showed a slight decrease in the interaction compared to that seen with the S73D mutant alone (data not shown). The same amounts of PIAS3 were bound to the protein A/G-coated beads. Similar results were obtained when anti-MITF antibody was prebound to protein A/G (data not shown).

Replacement of S409 in MITF with aspartate significantly reduced its transcriptional inhibition by PIAS3. In order to find out how phosphorylation of MITF regulates the inhibition of its transcriptional activity by PIAS3, we first determined the transcriptional activity of the mutated MITF in which aspartate was substituted for specific serines. NIH 3T3 fibroblasts, which do not abundantly express endogenous MITF, were cotransfected with a luciferase reporter plasmid containing the mMCP-6 promoter and pcDNA constructs of either MITF or MITF mutants. All the mutated MITF forms showed an increase in luciferase activity compared to the cells transfected with wild-type MITF (data not shown).

The role played by MITF phosphorylation in the suppression of its transcriptional activity by PIAS3 was then determined. NIH 3T3 cells were cotransfected with a luciferase reporter plasmid containing the mMCP-6 promoter and 0.1 μg of pcDNA constructs of either MITF or mutated MITF (S73D, S298D, or S409D) and with 0.1 μg of PIAS3 (Fig. 6). The levels of inhibition of transcriptional activity were similar when PIAS3 was cotransfected with either wild-type MITF or its S298D mutant (49% ± 2% and 42% ± 1%, respectively). A slight increase in the transcriptional inhibition of MITF by PIAS3 (69% ± 5%) was observed with the S73D MITF mutant. Almost no inhibition (5% ± 1%) in MITF transcriptional activity was observed when cells were cotransfected with the MITF mutant S409D and PIAS3. All of the results are expressed as the mean ± standard error of three experiments. Double mutation of MITF at S73 and S409 brought the transcriptional inhibition by PIAS3 into the same range as that achieved with the wild-type MITF; however, there was less inhibition by PIAS3 than by the S73D mutant alone.

DISCUSSION

Phosphorylation-dephosphorylation plays a major role in the assembly and disassembly of multiprotein complexes. As mentioned above, it was recently reported that GSK3β phosphorylates MITF on serine 298 (35). This serine phosphorylation enhanced the binding activity of MITF to the tyrosinase promoter. A mutation to proline at serine 298 has been identified in patients with Waardenburg syndrome type II (37). Furthermore, in activated melanocytes, an alanine substitution at either serine 73 or 409 produced transcriptionally inactive MITF, whereas MAPK and Rsk-1, which phosphorylate MITF at serines 73 and 409, respectively, promote its activation followed by its degradation (8, 40).

To evaluate the role played by MITF phosphorylation at serines at positions 298, 73, and 409 in its interaction with PIAS3, we constructed MITF mutants in which these serines were replaced by an aspartate, which mimics the charge brought by the phosphate group (11, 39). These constitutively phosphorylated mutants of MITF were cotransfected into NIH 3T3 fibroblasts with a luciferase reporter plasmid containing the mMCP-6 promoter, and the luciferase activity was enhanced in all three of the mutants. Aspartate substitution at serine 73 of MITF resulted in a slight increase in its association with PIAS3, whereas substitution at serine 409 almost totally abolished the capability of MITF to interact with PIAS3. These results strongly indicate that phosphorylation of MITF plays a role as an alternative transcriptional pathway, mediated either by the release of MITF from the PIAS3 inhibitor or by stronger interaction between the two proteins.

We further showed that RSK, which is known to phosphorylate MITF at position 409 (40), caused dissociation between MITF and PIAS3 and therefore a decrease in PIAS3-mediated MITF transcriptional activity. Moreover, when the S409A mutant, a mutant which has been found to be resistant to phosphorylation by RSK (23), was used, no change was observed in the PIAS3-MITF(S409A) association.

It has been reported that in melanocytes, replacement of Ser73 or Ser409 of MITF by alanine caused suppression of Kit ligand-mediated transcriptional activity (40). This suggests that phosphorylation at these serines is sufficient to signal ubiquitin-dependent proteolysis of this transcription factor (40). These results, together with our data, indicate that serine phosphorylation at these positions regulates MITF activity directly via transcriptional activation and MITF degradation and indirectly via its interaction with PIAS3.

MITF has several potential sites for protein-protein interaction in the bHLH-Zip motifs. The bHLH-Zip domain of MITF

is essential for the expression of the mMCP-6 gene (27), and the Zip domain of MITF has been found to be important for the development and differentiation of mast cells (24). In the present work, we demonstrated that the MITF Zip region is the domain responsible for direct interaction with PIAS3, which is a zinc finger protein (20). This Zip domain has recently been found to be responsible for the association of MITF with another zinc finger protein, myc-associated zinc finger-related factor (23). This association leads to the enhancement of MITF transcriptional activity (23). Previous work revealed that PIAS3 interacts directly with MITF and suppresses its transcriptional activity (18). The Zip domain of MITF contains several charged residues that upon phosphorylation play critical roles in the process of molecular recognition (33). Furthermore, this domain appears to be essential for interaction and DNA binding (9). Although serine 298 in MITF is located right after the Zip domain, we have focused in this work on the role of serines 73 and 409, which are not located in proximity to this domain, in the MITF-PIAS3 interaction (33). Thus, we assume that the phosphorylation on these two MITF serine residues induces conformational changes in MITF that allow the recruitment of some other proteins to the complex and thereby modulate the strength of the interaction.

The association between Jak-mediated phosphorylated STAT3 and PIAS3 has been thoroughly investigated (17). It is known that activation causes phosphorylation of STAT3, which then binds to PIAS3. We assume that MITF is similarly phosphorylated upon activation. In our colocalization experiments, we did indeed see greater nuclear interaction in cells activated for 10 min than in resting cells or cells activated for 30 min. However, we have also shown the inability of PIAS3 to associate with S409-phosphorylated MITF, which presumably mimics the phosphorylation that occurs upon cell activation. MAPK-mediated phosphorylation, which occurs at serine 73, has been shown to be maximal after 10 min of activation (40). In contrast, Rsk-1-mediated phosphorylation, which occurs at serine 409, was shown to be maximal at 20 min (40). This presumably explains why we observed increased colocalization of PIAS3 and MITF 10 min after activation, when most phosphorylation is occurring at S73 as opposed to S409, whereas 30 min after activation, both colocalization and coimmunoprecipitation of these proteins were reduced.

These results shed new light on the intracellular shuttling of PIAS3 from one cellular component to another. Moreover, we demonstrated a decrease in vivo in the interaction between MITF and PIAS3 in mast cells derived from $MITF^{di/di}$ mice, which lack the Zip domain in MITF (7). The interaction between the two proteins in cells derived from these mutated mice was not completely abolished, suggesting that there is some other protein(s) involved in this interaction. Furthermore, the slight increase in PIAS3-STAT3 association in cells derived from these MITF-truncated mice indicates that MITF plays a significant role in STAT3-mediated gene expression.

STAT3 is a latent transcription factor and a major molecule in the STAT family, which mediates cytokine- and growth factor-directed transcription. In a variety of hematopoietic derived cells, receptor stimulation leads to phosphorylation of tyrosine residues of STAT, which rapidly triggers DNA binding and STAT-mediated gene transcription (4, 16, 32). Interestingly, the binding of PIAS3 to STAT3 is induced following its phosphorylation, perhaps acting as a feedback mechanism preventing overactivation of this transcription factor (4). It was previously reported that STAT3 does not associate with PIAS3 that is already associated with MITF (18). Thus, while STAT3 phosphorylation leads to PIAS3 binding and inactivation of STAT3, the phosphorylation of MITF on serine 409 leads to the release of PIAS3, which can then potentially bind to STAT3. Thus, phosphorylation is a fine tuning of the regulation of the transcriptional machinery by the same inhibitor in a very different fashion. Further research will focus on whether the same motifs in PIAS3 are responsible for its association with STAT3 and MITF.

The oncogenic properties of constitutively active STAT3 offer an explanation for the ability of MITF to increase its transcriptional activity in melanoma (5). This activated STAT3 might induce the recruitment of PIAS3 into the STAT system and thus indirectly increase the transcriptional activity of MITF. This up regulation of MITF by STAT activation could be considered an indirect pathway for increasing transcriptional activity.

In conclusion, we suggest that the Zip region and phosphorylation of MITF at serine 409 play novel roles in the inhibition of activation of MITF transcriptional activity.

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