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# A New Role for the STAT3 Inhibitor, PIAS3

## A REPRESSOR OF MICROPHTHALMIA TRANSCRIPTION FACTOR*

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***In vitro* and *in vivo* evidence suggest that microphthalmia transcription factor (MITF) plays a key regulatory role in tissue-specific gene regulation in several cell types, including melanocytes, osteoclasts, and mast cells. A yeast two-hybrid search, using a portion of a nonmutated MITF gene as the bait in the screening of a mast cell library, resulted in the isolation of the STAT3 inhibitor, PIAS3. PIAS3 is a transcriptional inhibitor that acts by specifically inhibiting STAT3's DNA binding activity. We found that it can directly associate with MITF using an *in vitro* pull-down assay. Immunoprecipitation of MITF from rat basophilic leukemic cells or mouse melanocytes resulted in the specific co-immunoprecipitation of PIAS3. Co-transfection of MITF with PIAS3 in NIH 3T3 fibroblasts containing an mMCP-6 promoter-luciferase reporter demonstrated up to 94% inhibition of MITF-mediated transcriptional activation. Using a gel-shift assay, it was shown that PIAS3 can block DNA binding activity. It was also found that STAT3 does not interfere, either *in vitro* or *in vivo*, with the interaction between PIAS3 and MITF. These data suggest that PIAS3 functions *in vivo* as a key molecule in supressing the transcriptional activity of MITF, a role of considerable importance in mast cell and melanocyte development.**

The microphthalmia transcription factor (MITF)[1] is a basic helix-loop-helix leucine zipper (bHLH-Zip) DNA-binding protein (1). Mutation of the MITF gene, which resides at the *mi* locus in mice, results in deafness, bone loss, small eyes, and poorly pigmented eyes and skin (2). Mice strains harboring different mutations in the MITF gene have different phenotypes (3). One of these strains, *mi/mi* mice, have almost no mast cells (4). However, the transgenic *tg/tg* mice, which have an almost total lack of MITF expression (1), have a normal number of mast cells (5).

The role of MITF in gene regulation has been analyzed using two different approaches. One approach was the use of co-transfection assays and *in vitro* DNA binding assays, whereas the other approach utilized the available mouse models that harbor different genetic mutations in MITF. In 1996, Kitamura and his colleagues (6) demonstrated that the MITF protein in *mi/mi* mice is mainly located in the cytoplasm, and therefore they proposed that the presence of the mutated protein can lead to cellular defect by anchoring MITF hetrodimerization partners in the cytoplasm.

MITF regulates the expression of mouse mast cell protease (mMCP)-6 (7), mMCP-5 (8) c-*kit* (9), p75 nerve growth factor (8), granzyme B (10), tryptophan hydroxylase (11), and cathepsin K (12). Three MCP genes are down-regulated in mast cells not only from the *mi/mi* strain but also from the *tg/tg* strain (11). The MCPs have distinct expression patterns in mast cells from different environments, and important physiological functions have been demonstrated for some of them (13). Thus, their down-regulation in *tg/tg* mice demonstrates that MITF plays an important role in regulation of the expression of some of the most important mast cell-specific genes. MITF also regulates the transcription of the genes that encode tyrosinase (albino locus), tyrosinase-related protein, and pink-eyed Pmel 17 (silver) (14). Since the latter three genes control pigmentation, decreased expression of MITF in melanoma patients leads to diminished pigmentation.

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 (15–18). MITF also synergizes with PEBP2 to increase the rate of transcription of the mMCP-6 gene (19).

Since MITF is of global importance in mast cell development, we used a yeast two-hybrid screening approach to identify some of the proteins in this effector cell that interact with MITF (20). When a mast cell library was screened with a construct that encodes the bHLH-ZIP domain of MITF, only a few genes with very high affinity to the MITF gene were isolated. Not surprisingly, one of them was TFE3, the member of the MITF-TFE family closest to MITF. Another gene found to have high affinity to MITF was the protein kinase C-interacting protein 1 (PKCI), which is a ubiquitous member of the histidine triad family of proteins (21). We accumulated clear evidence that PKCI functions as a repressor of MITF-induced transcriptional activity (20). We demonstrated that the interaction between MITF and PKCI is negatively regulated by cell surface receptor engagement (20). This suggests that PKCI functions as a transcriptional repressor by association with MITF and prevention of MITF recruitment and/or activation of target genes until the appropriate stimulus is encountered.

Using the same yeast two-hybrid screening approach in the

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[1] The abbreviations used are: MITF, microphthalmia transcription factor; bHLH-Zip, basic helix-loop-helix leucine zipper; RBL, rat basophilic leukemic cells; PIAS3, protein inhibitor of activated STAT3; PKCI, protein kinase C-interacting protein 1; mMCP, mouse mast cell protease; GST, glutathione *S*-transferase; FITC, fluorescein isothiocyannate; PBS, phosphate-buffered saline.

present work, we have identified the STAT3 inhibitor, PIAS3, as being another gene with high affinity to MITF. All seven STAT proteins directly link cytokine receptor stimulation to gene transcription by acting as both cytosolic messengers and nuclear transcription factors. In unstimulated cells, a STAT protein exists in the cytoplasm as a monomer. Upon activation by tyrosine phosphorylation in response to ligand stimulation, STATs form dimers through SH2-phosphotyrosyl interactions (22). These dimers then translocate into the nucleus to activate transcription. In a yeast two-hybrid screen aimed at the identification of potential regulators of STAT1, a protein was identified which was later named PIAS1 (protein inhibitor of activated STAT1) (22). Four additional mammalian proteins related to PIAS1 were identified through data base searching and cDNA library screening. One of these proteins was PIAS3 (protein inhibitor of activated STAT3), which was found to be a novel protein (22). *In vivo* co-immunoprecipitation studies using specific antibody to PIAS3 indicated that PIAS3 interacts with STAT3 (23). Moreover, *in vitro* DNA binding analysis suggested that PIAS3 can block the DNA binding activity of STAT3 and, in addition, that PIAS3 inhibits STAT3-mediated gene activation (23).

In the present study, we present clear evidence of a function for PIAS3 as a repressor of MITF-induced transcriptional activity and show that STAT3 does not interfere either *in vitro* or *in vivo* with the interaction between PIAS3 and MITF.

## EXPERIMENTAL PROCEDURES

*Cells*—Melanocytes, B16S10.9 (20), RBL, and NIH 3T3 were maintained at 37 °C in growth medium, which was RPMI 1640 medium-supplemented with 2 mM L-glutamine, 2 mM nonessential amino acids, 100 units/ml penicillin, 100 $\mu$g/ml streptomycin (Invitrogen), 50 $\mu$M $\beta$-mercaptoethanol (Fisher Scientific, Medford, MA), and 10% fetal calf serum.

*Plasmid Construction*—The bait was constructed in pAS2-1 by ligation of PCR-amplified cDNA covering the MITF cDNA fragment coding the sequence of 608–1113 (bHLH-Zip) into the *Bam*HI and *Pst*I sites for use in the yeast two-hybrid screening. The cDNA library was prepared from resting RBL cells mRNA by reverse transcription and cloned in the *Eco*RI site of the pGAD10 vector. The insert size was on average 1.7 kb and ranged from 0.3 to 3.5 kb. The cDNA encoding the open reading frame of PIAS3 was subcloned into the *Xba*I and *Hin*dIII sites of the pcDNA3.1(−) vector (Invitrogen). pGEX4T-1-PIAS3 was kindly provided by Dr. Shuai (University of California, Los Angeles). STAT3 pcDNA was kindly provided by Dr. Zipori, Weizmann Institute, Rehovot, Israel. Normal mouse MITF (1129 bp) was inserted into the pGEX-4T-3 vector (Amersham Biosciences, Inc.). An amplification was made from the nonmutated mouse MITF. 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 mouse mMCP-6 gene (−191 to +26), as well as a construct with a deleted MITF binding site (−151 to +26) (7), were generously provided by Prof. Kitamura, Osaka University Medical School, Osaka, Japan.

*Yeast Two-hybrid Experiments*—Yeast transformation and two-hybrid screening and assays were performed according to the instructions for the MATCHMAKER two-hybrid system (CLONTECH) (20). Yeast CG1945 was transformed with pAS2-1 MITF (bHLH-Zip) to screen for proteins that were capable of interacting with MITF. We screened the pGAD10 RBL cDNA library expressed in yeast and selected the transformed yeast by growth on synthetic medium lacking Trp, Leu, and His, and containing 0.25 mM 3-aminotriazole. Five days later one of the selected colonies was regrown on the same medium. The doubly selected colonies were then tested for galactosidase activity. The cDNA of positive clones was rescued in *Escherichia coli* DH5$\alpha$ and subsequently sequenced.

*In Vitro GST Pull-down Assay*—GST fusion protein MITF was expressed in protease-deficient *E. coli* strain B12 and purified on gluthatione-Sepharose beads (Amersham Biosciences, Inc.) essentially as described before (20). Pull-down assays (24) were performed with GST-MITF fusion protein (1 $\mu$g-5 $\mu$g) bound to Sepharose beads and preincubated for 1 h at 4 °C in 1 ml of binding buffer (100 mM KCl, 20 mM Hepes, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 0.1% Nonidet P-40). 1–10 $\mu$l of $^{35}$S-labeled PIAS3 or STAT3 proteins, synthesized using the TNT-coupled rabbit reticulocyte lysate system (Promega), were added to each preincubation mix and the binding reaction carried out overnight at 4 °C. Beads were washed four times in 1 ml of PBS/290 mM NaCl, boiled for 7 min in sample buffer, and aliquots were examined by electrophoresis. Integrity and quantity of GST fusions were confirmed by Gelcode Bluestain reagent (Pierce), and autoradiography detected the amount of retained PIAS3 or STAT3.

*Transient Co-transfection Luciferase Assay*—NIH 3T3 cells ($5 \times 10^5$) were transfected utilizing a liposomal delivery system (Transfast, Promega) with 1 $\mu$g of reporter, 0.25 $\mu$g of pcDNA-MITF, and various concentrations of pcDNA3.1 alone as a nonspecific control or with pcDNA-PIAS3 or pcDNA-STAT3 as the experimental variables. $10^5$ cells/well were incubated in six-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 co-transfection of the reporter with pcDNA alone. The ratio was expressed as the relative luciferase activity.

*Gel-shift Assays*—Probes were double-stranded oligonucleotides of 25 bp that spanned the E-boxes of the mMCP-6 promoter as described in Ref. 8. Probes were end-labeled using DNA polymerase I large fragment (Klenow, MBI Fermentas, St. Leon-Rot, Germany). Binding reactions (20 $\mu$l) contained 100,000 cpm labeled probe, 1 $\mu$g of poly(dI-dC) (Amersham Biosciences, Inc.), 10% glycerol, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 10 mM Hepes, and 2 $\mu$g of GST-MITF with or without increasing doses of GST-PIAS3 (2–4 $\mu$g) or GST as control. Reactions were incubated without the probe on ice for 90 min, and then the labeled probe was added, and the reaction was incubated at room temperature for 30 min and resolved on a 6% TBE (10% Tris, 5.5% boric acid, 20 mM EDTA)-polyacrylamide nondenaturating gel (25).

*Immunoprecipitation*—RBL cells were lysed by the addition of 250 $\mu$l of cold lysis buffer (0.01 M Tris-HCl, pH 7.4, 1% deoxycholate, 1% Triton X-100, 0.1% SDS, 0.15 M NaCl, and 0.25 $\mu$M phenylmethylsulfonyl fluoride) and 10 $\mu$l of protease inhibitor mixture (Sigma). Cells were then homogenized and their supernatants collected after 15-min centrifugation in a microcentrifuge at 4 °C. Recovered lysates were incubated with anti-mouse MITF antibody (20), anti-mouse PIAS3 antibody (Santa Cruz), or anti-mouse STAT3 antibody (Santa Cruz), prebound to 10 mg of protein A-agarose (Invitrogen), and incubated with agitation overnight at 4 °C. Recovered immune complexes were washed three times with lysis buffer (1:2) and then washed once with Tris-EDTA washing buffer. All buffers contained protease inhibitors. Proteins were solubilized in Laemmli sample buffer containing 0.5% SDS.

*Gel Electrophoresis and Western Blots*—Proteins were resolved by 10% SDS-PAGE under reducing conditions and transferred to 0.45-$\mu$m nitrocellulose membranes. Visualization of reactive proteins was by enhanced chemiluminescence (20).

*Indirect Fluorescent Immunocytochemistry*—Cells were grown on glass coverslips in six-well plates. After extensive washing with PBS, the cells were fixed with 1.5 ml of 3.7% formaldehyde in PBS for 10 min. The 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 45-min blocking with normal donkey serum, indirect fluorescent immunocytochemistry was carried out using rabbit anti-MITF (18) or goat anti-PIAS3 (Santa Cruz) antibodies and Cy5-conjugated donkey anti-rabbit IgG or FITC-conjugated rabbit anti-goat IgG (Jackson ImmunoResearch), respectively. Background staining was examined using the appropriate secondary antibody alone. Specimens were examined, analyzed, and photographed by laser scanning confocal microscopy using the Zeiss LSM 410 confocal laser scanning system connected to a Zeiss Axiovert 135M microscope with 63×/1.2 C-Apochromat water immersion lens (Zeiss, Jena, Germany). Green fluorescence of FITC and Cy5 labels was excited with argon laser (488 nm excitation line with 515-nm-long pass barrier filter).

## RESULTS

*Association of MITF and PIAS3*—We used the yeast two-hybrid system to screen for MITF-interacting proteins, as described previously (20). Quiescent RBL cells were used to produce the cDNA library that was screened; thus the proteins found to associate with MITF did not include any of those whose mRNA expression is induced in activated cells. One of the isolated cDNA clones encoded the PKCI protein, whereas another isolated cDNA encoded the PIAS3 protein.

The direct association between full-length MITF and PIAS3 was determined using an *in vitro* pull-down assay. MITF was


FIG. 1. **PIAS3 binds MITF *in vitro*.** $^{35}$S-Labeled PIAS3 was incubated with GST-MITF immobilized on glutathione-Sepharose beads, and, after washing, retained $^{35}$S-labeled PIAS3 protein was examined by SDS-PAGE and autoradiography. One representative of three experiments is shown.

expressed in bacteria as GST fusion, immobilized on glutathione-agarose beads, and assayed for its ability to retain *in vitro* translated PIAS3 labeled with [$^{35}$S]methionine. PIAS3 bound to GST-MITF but not to control GST alone. Thus, PIAS3 and full-length MITF can complex with each other both in yeast and *in vitro* (Fig. 1).

To obtain evidence of the possible existence *in vivo* of an MITF-PIAS3 complex, we then investigated whether immunoprecipitation of MITF from quiescent RBL cells or melanocytes would result in specific co-immunoprecipitation of PIAS3. This was carried out by incubating the RBL lysates with either anti-MITF or anti-PIAS3 antibody prebound to protein A-agarose beads. The recovered and resolved immune complexes showed the co-immunoprecipitation of PIAS3 with MITF using either immunoprecipitation approach (Fig. 2). Thus, PIAS3 forms a complex with MITF in mast cells and melanocytes.

Next we determined whether MITF could form a complex with PIAS3 and STAT3 *in vivo*. First we examined whether *in vitro* translated STAT3 could associate directly with GST-MITF fusion protein immobilized on glutathione-agarose beads. The results presented in Fig. 3 clearly show that STAT3 does not bind to MITF. Furthermore, in another set of *in vitro* pull-down assays, we demonstrated that STAT3 could not bind to the PIAS3-MITF complex (Fig. 4). Immunoprecipitation of MITF from quiescent RBL cells resulted in specific co-immunoprecipitation of PIAS3, but not STAT3. Thus it seems that, at least in quiescent cells, a complex containing MITF, PIAS3, and STAT3 does not exist.

The gel-shift assay was performed to determine whether PIAS3 can modulate the binding of MITF to E-boxes in the mMCP-6 promoter (7) using GST-MITF and GST-PIAS3 fusion proteins (Fig. 5). A marked decrease in MITF binding to the oligonucleotides was observed when the GST-MITF was preincubated with increasing doses of GST-PIAS3. Neither GST-PIAS3 or GST alone showed any binding to this oligonucleotide. These results suggest that PIAS3, by its association with MITF, decreases MITF's binding to DNA.

*Regulation of MITF Transcriptional Activity by PIAS3*—To test the effect of PIAS3 interaction on MITF transcriptional activity, we chose to work with cells that did not abundantly express endogenous MITF. We have previously shown that MITF is not present in NIH 3T3 fibroblasts (20). NIH 3T3 fibroblasts were co-transfected with a luciferase reporter plasmid containing the mouse mMCP-6 promoter and pcDNA constructs of either MITF alone or MITF and PIAS3. Up to 94% inhibition of the luciferase activity was observed when the NIH 3T3 fibroblasts were co-transfected with 0.25 $\mu$g of MITF and 1 $\mu$g of PIAS3 (Fig. 6). Moreover, the extent of inhibition increased with increasing concentrations of PIAS3. Co-transfection of MITF with the pcDNA vector alone showed no inhibitory effect on the MITF-induced luciferase activity.

To test the effect of STAT3 on the suppression of MITF transcriptional activity by PIAS3, NIH 3T3 fibroblasts were co-transfected with a luciferase reporter plasmid containing the pcDNA constructs of either MITF or MITF and PIAS3 and increasing concentrations of STAT3 (Fig. 7). STAT3 did not show any effect on the inhibition of MITF transcriptional activity caused by PIAS3 in quiescent cells.

*Intracellular Localization of PIAS3*—We have previously

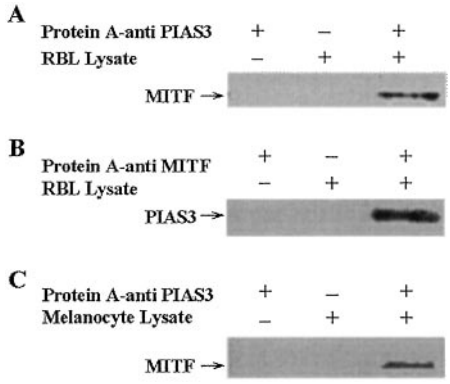


FIG. 2. **Association of MITF with PIAS3 in RBL cells and melanocytes.** Western blot of immunoprecipitation of PIAS3 with MITF from RBL cells (*A*, *B*) and melanocytes (*C*). Immunoprecipitation from lysates of RBL cells and mouse melanocytes with either anti-PIAS3 (*A*, *C*) or anti-MITF (*B*) protein A-agarose beads. Blots were probed with either anti-MITF (*A*, *C*) or anti-PIAS3 (*B*) antibodies. One representative of three experiments is shown.

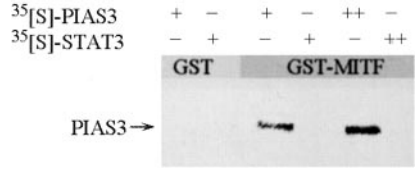


FIG. 3. **PIAS3 but not STAT3 binds MITF *in vitro*.** $^{35}$S-Labeled PIAS3 or $^{35}$S-labeled STAT3 (+, 15 ng; ++, 30 ng) were incubated with either GST or GST-MITF immobilized on glutathione-Sepharose beads, and, after washing, retained $^{35}$S-labeled PIAS3 or $^{35}$S-labeled STAT3 protein were examined by SDS-PAGE and autoradiography.

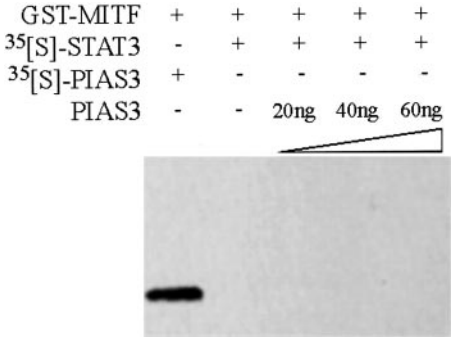


FIG. 4. **STAT3 does not bind *in vitro* to PIAS3-MITF complex.** $^{35}$S-Labeled PIAS3 or $^{35}$S-labeled STAT3 (15 ng) was incubated with GST-MITF immobilized on glutathione-Sepharose beads with or without increasing concentrations of PIAS3. After washing, retained $^{35}$S-labeled PIAS3 or $^{35}$S-labeled STAT3 protein was examined by SDS-PAGE and autoradiography.

shown that MITF is localized in the nucleus of RBL cells (20). The intracellular localization of MITF and PIAS3 was determined by indirect fluorescent immunocytochemistry. For MITF localization, cells were labeled with Cy5-conjugated secondary antibody (red) that recognizes the highly specific anti-MITF antibody. For PIAS3 localization, the cells were labeled with FITC-conjugated secondary antibody (red) that recognizes the highly specific anti-PIAS3 antibody. Cells stained with anti-MITF showed mostly nuclear staining (Fig. 8*A*). All cells had PIAS3 both in the cytosol and in their nuclei (Fig. 8*B*). Normal donkey serum staining showed practically no background staining.

The presence of both PIAS3 and MITF in the nucleus corroborates the results from the co-transfection experiments that suggest that PIAS3 serves as a repressor of MITF transcriptional activity.

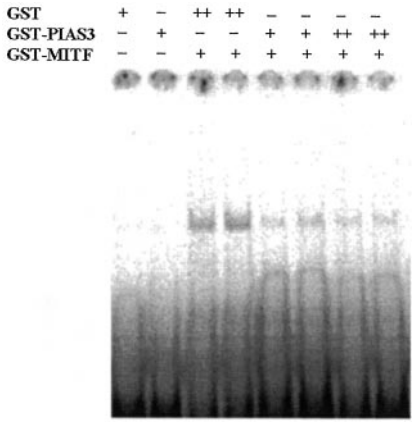


FIG. 5. **PIAS3 inhibits MITF DNA binding.** GST-MITF was incubated with or without GST or increasing concentrations of GST-PIAS3 (+, 2 $\mu$g; ++, 4 $\mu$g) in combination with $^{32}$P-labeled oligonucleotide probe corresponding to the mMCP-6 promoter (7). One representative of three experiments is shown.

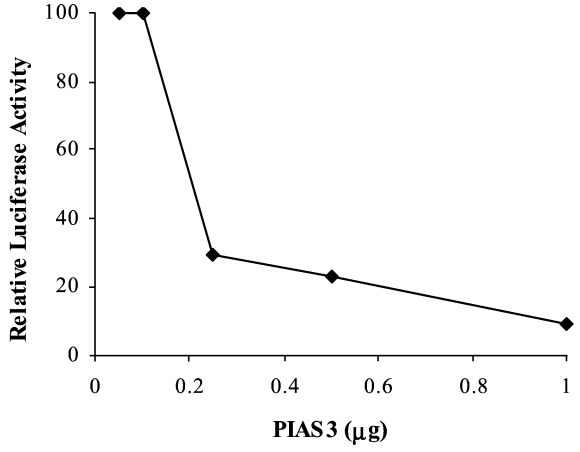


FIG. 6. **Inhibition of MITF transcriptional activity by PIAS3.** Luciferase reporter plasmid, containing a promoter region of the mMCP-6, was co-transfected with either MITF or MITF and PIAS3, and the relative MITF transcriptional activity was determined. The luciferase activity was normalized by total protein concentration. The normalized value was divided by the activity obtained by co-transfection of the mMCP-6 luciferase-reporter construct and the pcDNA (vector control) and was expressed as relative luciferase activity. One representative experiment out of three is shown.

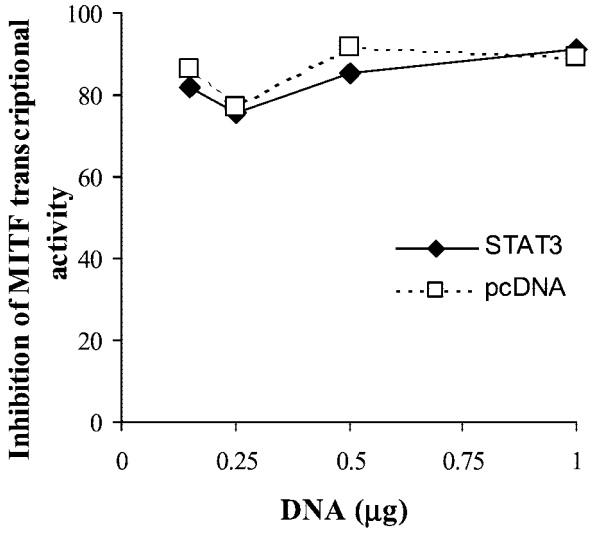


FIG. 7. **The effect of STAT3 on the inhibition of MITF transcriptional activity by PIAS3.** Luciferase reporter plasmid, containing a promoter region of the mMCP-6, was co-transfected with either 0.25 $\mu$g of MITF or 0.25 $\mu$g of MITF and 0.25 $\mu$g of PIAS3 and increasing concentrations of STAT3. The relative MITF transcriptional activity was determined as described above. One representative experiment of three is shown.

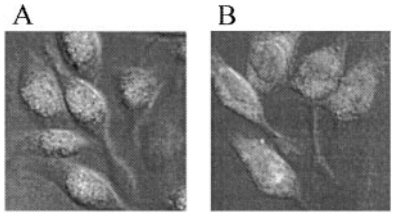


FIG. 8. **Intracellular localization of PIAS3 and MITF in RBL cells.** RBL cells were stained with either anti-MITF and Cy5-conjugated donkey anti-rabbit IgG (*A*) or anti-PIAS3 and FITC-conjugated rabbit anti-goat IgG (*B*). Cells showed nuclear staining with anti-MITF and cytosolic and nuclear staining with anti-PIAS3. One representative experiment of three is shown.

## DISCUSSION

MITF has been shown to be capable of binding to several transcription factors, including c-Fos (26), USF2 (18), and TFE3 (which also associates with MITF in the yeast two-hybrid screening of mast cells (20)). In addition, we have described the binding of PKCI to MITF in quiescent cells (20). Now we show that PIAS3 can interact with MITF and inhibit its transcriptional activity.

Evidence supporting the idea that PIAS3 is a physiological regulator of MITF is presented in this article. PIAS3 was isolated from the yeast two-hybrid cDNA library using truncated wild type MITF as the bait. It bound to full-length MITF in a pull-down assay *in vitro*. Moreover, MITF and PIAS3 were found as a complex in quiescent RBL cells and melanocytes in co-immunoprecipatation assays. Using the ability of MITF to activate the transcription of a reporter gene under the control of the MCP-6 gene, we demonstrated that PIAS3 inhibits MITF function *in vivo*.

The expression of mMCP-6 is highly restricted to mast cells (27), and even fibroblasts in normal tissue do not express this protease (28). mMCP-6 has been shown to be a very potent chemoattractant for neutrophils, suggesting that it plays an important function during many inflammatory processes (29). Kitamura and his group (7) have also shown that a short sequence from the MCP-6 promoter contains an MITF response element. Thus the study of MITF activity through experiments performed with the MCP-6 promoter is highly relevant for a better understanding of MITF regulation in mast cells.

PIAS3 inhibited the MCP-6 promoter controlled transcription in a dose-dependent manner. The addition of recombinant PIAS3 to MITF and the target oligonucleotide resulted in the decreased binding of MITF containing complexes to DNA and not in the appearance of slower migrating complexes containing DNA bound to MITF and PIAS3 in gel retardation assays. This showed that the inhibition of MITF transcription is a result of decreased DNA binding of MITF caused by PIAS3 and not by the binding of an inactive complex containing MITF and PIAS3 to a response element.

Although MITF has been shown to have key regulatory roles both in mast cells and in melanocytes (2), the role played by STAT3 in receptor-mediated signaling has been poorly reported in mast cells. STAT3 is a latent transcription factor that mediates cytokine- and growth factor-directed transcription. In a variety of hematopoietic-derived cells, receptor stimulation leads to phosporylation of tyrosine residues of STAT, which rapidly triggers DNA binding and STAT-mediated gene transcription (22, 23, 30). Our data strongly suggest that STAT3 does not associate with PIAS3 that is already associated with

MITF. Co-transfection of MITF with increasing concentrations of PIAS3 caused dose-dependent repression of MITF transcriptional activity. However, co-transfection of MITF, PIAS3, and STAT3 did not affect the inhibition of MITF transcriptional activity by PIAS3. Thus it seems that the binding of PIAS3 *in vivo* to MITF might occur under circumstances in which its binding to STAT3 is minimal.

The network of transcription factors involved in mast cell regulation is very complex. Here our results point toward two new participants in this network, PIAS3 and STAT3. Understanding the circumstances and mechanisms involved in the regulation of STAT3 and MITF by PIAS3 is our next research goal.

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