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Identification of the Region of *mi* Transcription Factor Which Is Responsible for the Synergy with PEBP2/CBF

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The *mi* locus encodes the *mi* transcription factor (MITF), a member of the basic-helix-loop-helix-leucine zipper protein family of transcription factors. MITF binds the αB1/AML1 subtype of the α subunit of the polyomavirus enhancer binding protein 2 (PEBP2). These two transcription factors synergistically transactivate the mouse mast cell protease 6 (MMCP-6) gene. The interaction of PEBP2 with MITF is mediated through the region carboxy-terminal to the DNA-binding Runt domain. In the present study, we examined the region of MITF that is responsible for the interaction with PEBP2. The MITF mutant that lacked the region aa 67–152 did not bind PEBP2, and the mutant that lacked the region aa 1–152 lost the synergistic function in the transactivation of the MMCP-6 promoter. We conclude that the region amino-terminal to the basic region of MITF is required for physical and functional interactions with PEBP2.

The *mi* locus of mice encodes a member of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) protein family of transcription factors (hereafter called *mi*-transcription factor, MITF) (1, 2). The MITF encoded by the mutant *mi* allele deletes 1 of 4 consecutive arginines in the basic domain (hereafter *mi*-MITF) (1–3). The *mi*-MITF is defective in the DNA binding activity and the nuclear localization potential, and it does not transactivate target genes (4–12). The *mi*/*mi* mice show microphthalmia, depletion of pigment in both hair and eyes, osteopetrosis, and decrease of mast cells (13–16). In addition to the decrease in number, the phenotype of mast cells is abnormal in *mi*/*mi* mice (17–21). We have demonstrated the involvement of MITF in transactivation of various genes in mast cells, such as mouse mast cell protease-6 (MMCP-6) (5), c-*kit* (7), MMCP-5 (8), p75 nerve growth factor receptor (9), Granzyme B, tryptophan hydroxylase (10), integrin α4 subunit (11), and MMCP-4 genes (12).

We previously analyzed the mechanism of transactivation by MITF in the MMCP-6 gene, and found that the GACCTG motif in its promoter region mediated the transactivation by MITF (5). We have recently reported that this GACCTG motif partly overlapped with the TGTGGTC motif which is recognized by the polyomavirus enhancer binding protein 2 (PEBP2), and that PEBP2 and MITF synergistically transactivated the MMCP-6 promoter (22). PEBP2, also termed the core-binding factor (CBF), is composed of α and β subunits. The α subunit of PEBP2 has a DNA binding domain called the Runt domain (23) and MITF interacts with the region carboxy-terminal to the Runt domain which harbors the transactivation domain (22, 24). PEBP2 interacts with various types of transcription factors, such as Ets-1 (25–28), Myb (29, 30), and C/EBP (31, 32) through overlapping regions in the C-terminus downstream from the Runt domain. In contrast to PEBP2, only a few factors are known to interact with MITF. To date, the related bHLH-Zip proteins, TFE3, TFEB and TFEC, all of which are highly homologous with MITF, were reported to bind MITF (3). To our knowledge, PEBP2 was the first transcription factor that bound MITF and that does not belong to the bHLH-Zip protein family. In the present study, we examined the domain of MITF which mediated the physical and functional interaction with PEBP2. In contrast to interactions with other bHLH-Zip proteins, the region of MITF amino-terminal to the basic region was necessary for the physical interaction with PEBP2, and the deletion of this region abolished the synergistic activation of the MMCP-6 promoter.

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Abbreviations used: MITF, *mi* transcription factor; bHLH-Zip, basic-helix-loop-helix leucine zipper; PEBP2, polyomavirus enhancer binding protein; MMCP-6, mouse mast cell protease 6; CBF, core-binding factor; GST, glutathione-S-transferase; MAPK, mitogen-activated protein kinase.

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MATERIALS AND METHODS

Plasmids. The whole coding region of MITF had been cloned into Bluescript KS(−) plasmid (pBS; Stratagene, La Jolla, CA) in our laboratory (hereafter called pBS-MITF) (4). The N-terminal, C-terminal or internal deletion constructs of MITF were produced by PCR using pBS-MITF as template. Some deletion constructs were subcloned into pGEX vectors (Amersham-Pharmacia Biotech, Uppsala, Sweden) for production of GST fusion proteins. pBS-PEBP2αB containing the whole coding region of PEBP2αB was reported previously (22). A part of PEBP2αB cDNA corresponding to aa 177 to 341 was amplified by PCR, and subcloned into pGEX vector to produce GST-PEBP2αB. The pEF-BOS expression vector was kindly provided by Dr. S. Nagata (Osaka University Medical School, Osaka) (33). The *Sma*I-*Hinc*II fragment of pBS-MITF was introduced into the *Xba* I site (blunted) of pEF-BOS to produce BOS-MITF. The expression plasmids containing deletion mutants of MITF were constructed by PCR. The expression plasmid containing the coding region of PEBP2αB and the reporter luciferase construct containing the tetrameric fragments of the GACCTG and TGTGGTC motifs were reported previously (22). All of the PCR products were verified by sequencing.

Purification of recombinant proteins and in vitro binding assays. The various deletion mutants of MITF and PEBP2αB were expressed in *Escherichia coli* as GST fusion proteins, and immobilized on glutathione-agarose beads according to the manufacturer's instructions (Amersham-Pharmacia Biotech). The *in vitro*-translated and ^{35}S-methionine-labeled PEBP2αB and MITF mutant proteins were prepared using the TNT system (Promega, Madison, WI). GST-fusion proteins (2 μg) immobilized on glutathione-agarose beads were incubated with *in vitro*-translated and ^{35}S-labeled proteins for 1 hour at room temperature with gentle rotation. The beads were washed four times with the washing solution (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.5% NP-40) and subjected to SDS-PAGE on a 10% acrylamide gel.

Transfection and luciferase assays. Jurkat T-cell leukemia cell line was maintained in RPMI 1640 medium supplemented with 10% FCS. The luciferase reporter (6 μg) and effector plasmids were co-transfected into Jurkat cells by electroporation (260 V/975 μF). The amounts of effector plasmids used were: BOS-MITF, 2 μg; BOS-PEBP2αB, 9 μg; and BOS-PEBP2β, 3 μg. The expression vector containing β-galactosidase gene (1 μg) was also transfected simultaneously to examine the transfection efficiency. Cells were harvested 24 hours after transfection and lysed with 0.1 M potassium phosphate buffer (pH 7.4) containing 1% Triton X-100. Soluble extracts were then assayed for luciferase activity with a luminometer (LB96P, Berthold GmbH, Wildbad, Germany) and for β-galactosidase activity. The luciferase activity was normalized with the β-galactosidase activity and protein content. Each luciferase activity was divided by the value obtained with transfection of the reporter plasmid and an empty pEF-BOS instead of expression plasmids, and was shown as relative luciferase activity.

Electrophoretic gel mobility shift assay (EGMSA). The production and purification of GST fusion proteins were described previously (4). The oligonucleotide (5′-CCACCGTGTGACCTGTGGTCATCA) was used as a probe (The GACCTG motif was underlined). The oligonucleotide was labeled with α-^{32}P-dCTP by filling 5′-overhangs. DNA-binding assays were performed in a 20 μl reaction mixture containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 75 mM KCl, 1 mM dithiothreitol, 4% Ficoll type 400, 50 ng of poly (dI-dC), 25 ng of labeled DNA probe, and 3.5 mg of GST fusion proteins. After the incubation at room temperature for 15 minutes, the reaction mixture was subjected to electrophoresis on a 5% polyacrylamide gel in 0.25 × TBE buffer (1 × TBE is 90 mM Tris-HCl, 64.6 mM boric acid, and 2.5 mM EDTA, pH 8.3). The gels were dried on Whatmann 3MM chomatography paper and subjected to autoradiography.

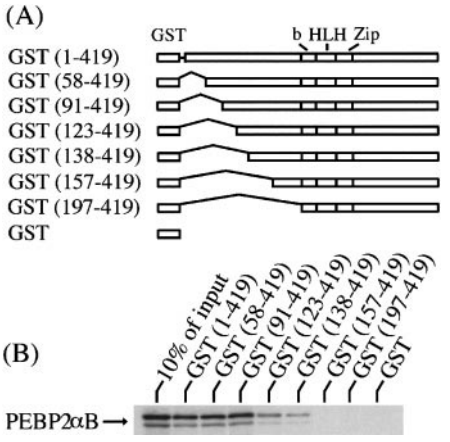

FIG. 1. Physical interaction of PEBP2αB with GST-MITF mutants. (A) Diagram of GST-MITF mutants that were truncated from the N-terminus and used for *in vitro* binding experiments. (B) PEBP2αB was ^{35}S-labeled and subjected to coprecipitation with GST, GST-MITF, or GST-MITF mutants bound to glutathione agarose beads. (MITF(1–419) represents the full length MITF.

RESULTS

The region required for physical interaction with PEBP2. To identify the region required for the physical interaction with PEBP2, we carried out in vitro binding experiments by using various deletion mutants of MITF. Of the three isoforms of the PEBP2α subunit (23), we analyzed PEBP2αB, since MITF showed transcriptional synergy with PEBP2αB but not with other two isoforms, PEBP2αA and PEBP2αC (22). First, various GST fusion proteins containing the progressive N-terminal deletion mutants of MITF were produced (Fig. 1A, MITF(1–419) is the full length MITF protein). The ^{35}S-labeled PEBP2αB was subjected to coprecipitation with GST or GST-MITF fusion proteins immobilized on glutathione-agarose beads, and the protein complexes were analyzed by SDS-PAGE. GST-MITF(1–419) but not GST bound PEBP2αB (Fig. 1B). Among the GST-MITF mutants, GST-MITF(58–419) and GST-MITF(91–419) bound PEBP2 αB at levels comparable to GST-MITF(1–419) (Fig. 1B). The amounts of PEBP2αB bound to GST-MITF(123–419) and GST-MITF(138–419) were smaller than that bound to GST-MITF(1–419). In contrast, neither GST-MITF(157–419) nor GST-MITF(197–419) bound PEBP2αB (Fig. 1B).

We next examined the binding of variously deleted and ^{35}S-labeled MITF to GST-PEBP2αB (Fig. 2A). We used the GST fusion protein that contained the region downstream from the Runt domain of PEBP2αB (aa 177–341) since we have found that this region was important for the interaction with MITF (22). GST-PEBP2αB bound the full length MITF(1–419) and all the C-terminal deletion mutants of MITF examined (Fig. 2B). We were unable to synthesize further C-terminal deletions probably because of the instabil-

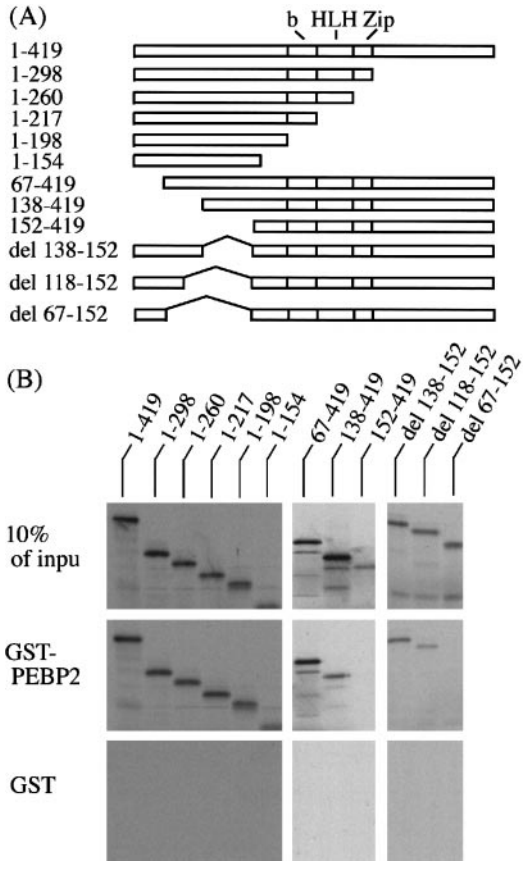

FIG. 2. Physical interaction of MITF mutants with GST-PEBP2. (A) Diagram of various deletion mutants of MITF used for *in vitro* binding experiments. (B) The MITF mutants were ^{35}S-labeled and subjected to coprecipitation with GST or GST-PEBP2αB. Ten percent of input materials are also shown.

ity of the products (data not shown). We then examined the physical interaction of the progressive N-terminal deletion mutants of MITF with PEBP2αB. The amount bound to GST-PEBP2αB was decreased with MITF(138–419) and no binding was observed with MITF(152–419). To see the effect of the region deleted in this construct, we prepared the internal deletion mutants of MITF and examined their bindings to GST-PEBP2αB. The binding slightly decreased with the MITF mutant lacking aa 138–151, and significantly decreased with the MITF mutant lacking aa 118–151. GST-PEBP2αB did not bind to the MITF mutant lacking aa 67–151 at all (Fig. 2B).

The region required for the functional synergy with PEBP2αB. MITF and PEBP2 synergistically transactivate MMCP-6 promoter through the GACCTG and TGTGGTC motifs, respectively (22). Various MITF mutants were examined to identify the region required for the functional synergy with PEBP2. The tetrameric fragments containing the GACCTG and TGTGGTC motifs were cloned into the plasmid carrying the minimal MMCP-6 promoter (22), and was used as the reporter plasmid. Jurkat cells were transfected with the reporter and plasmids encoding MITF mutants and PEBP2αB. The plasmid encoding PEBP2β was also transfected since PEBP2αB normally functions with PEBP2β in vivo (24). The synergy with PEBP2 was observed with the full-length MITF(1–419) (Fig. 3) as reported previously (22). MITF (1–298), which lacked the region downstream from the zipper domain, showed a synergy comparable to MITF(1–419). Among the N-terminal deletion constructs, synergy was detected with MITF(67–298) at a level comparable to MITF(1–419), and with MITF(138–298) at a reduced but still significant level. However, the synergy between MITF(152–298) and PEBP2 was hardly detectable (Fig. 3).

MITF binds DNA and shows the transactivation ability as a homo-dimer (34). There is a possibility that the reduction or abolishment of the synergy with PEBP2 might be due to the loss of dimerization ability of MITF (138–298) or MITF (152–298). To exclude this

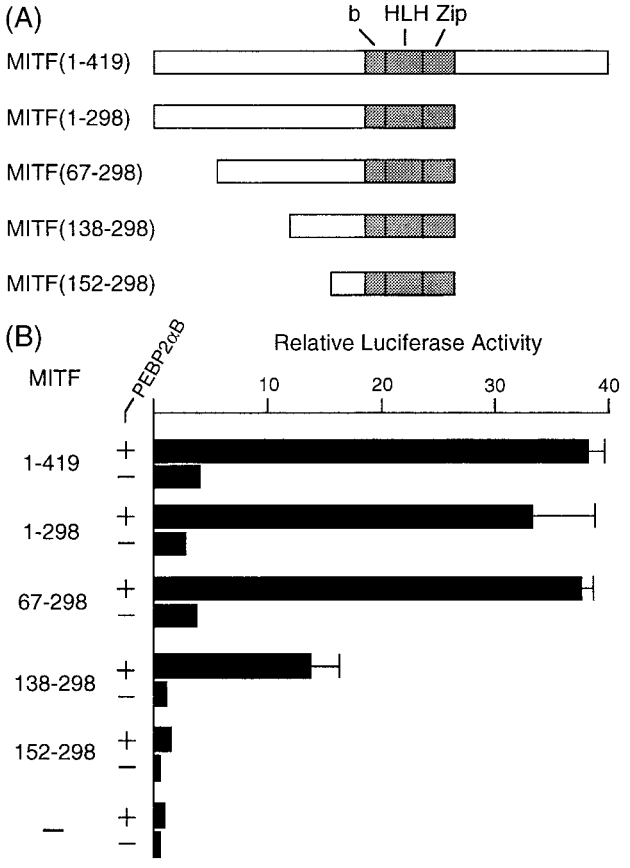

FIG. 3. Synergistic transactivation of the MMCP-6 promoter by PEBP2 and MITF mutants. (A) Diagram of the truncated MITF mutants used for the luciferase reporter assays. (B) The luciferase reporter containing the tetrameric GACCTG and TGTGGTC motifs from the MMCP-6 promoter was transfected into Jurkat cells with effector plasmids encoding PEBP2 (PEBP2αB + PEBP2β) and the MITF mutants as indicated. Bars represent means ± standard errors (SE) of three experiments.

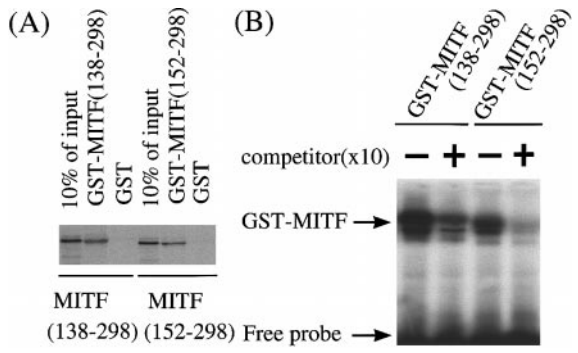

FIG. 4. Dimerization and DNA binding ability of MITF (138–298) and MITF (152–298). (A) MITF (138–298) was 35S-labeled and subjected to coprecipitation with GST or GST-MITF (138–298) bound to glutathione agarose beads. MITF (152–298) was also examined in a similar manner. (B) The binding of MITF (138–298) or MITF (152–298) to the GACCTG motif was examined by EGMSA.

possibility, the dimerization ability of MITF (138–298) or MITF (152–298) was examined. The ^{35}S-labeled MITF (138–298) bound GST-MITF (138–298) coated beads but not GST coated beads. The ^{35}S-labeled MITF (152–298) also specifically bound GST-MITF (152–298) (Fig. 4A). We next examined the DNA binding ability of MITF (138–298) or MITF (152–298) by EGMSA. GST-MITF (138–298) or GST-MITF (152–298) bound the oligonucleotide containing the GACCTG motif located in the MMCP-6 promoter (Fig. 4B). The excess amount of the oligonucleotide with the GACCTG motif competed for the binding of GST-MITF (138–298) or GST-MITF (152–298) (Fig. 4B). The excess amount of the oligonucleotide mutated in the GACCTG motif (GACCTG to GTCCAG) did not compete for the binding of GST-MITF (138–298) or GST-MITF (152–298), and no retarded band was detected by the addition of GST alone (data not shown). These results showed that the dimerization ability was not abolished in MITF (138–298) or MITF (152–298). This strongly suggested that the reduction or abolishment of synergy might be due to the impaired interaction with PEBP2 in MITF (138–298) or MITF (152–298).

DISCUSSION

PEBP2 binds MITF and these two factors synergistically transactivate the MMCP-6 promoter (22). The region of PEBP2αB (aa 177–341) C-terminal to the Runt domain is required for both the physical and the functional interactions with MITF (22). In the present study, we investigated the region of MITF that is responsible for these interactions with PEBP2. Both luciferase reporter and physical interaction studies showed that the essential region lies N-terminally to the bHLH-zip region. In interaction assays, the bHLH-Zip and the following C-terminal regions did not interact with PEBP2. Also in luciferase assays, the region C-terminal to the bHLH-Zip region was totally dispensable for the synergistic transactivation with PEBP2. The involvement of the bHLH-Zip region in this synergy could not be addressed because the region is essentially required for the dimerization and DNA-binding of MITF. Further dissection of the region N-terminal to the bHLH-Zip region revealed the importance of two adjacent subregions, which were aa 67–137 and aa 138–151. When these two regions were sequentially deleted with progressive N-terminal truncation of MITF, the synergistic transactivation with PEBP2 exhibited stepwise reductions and was abolished. The similar stepwise reduction was observed for the physical interaction in GST pull down assays. The region aa 152–419 did not interact with PEBP2 by itself. Attachment of the regions aa 138–151 and aa 67–151 to this region enabled the constructs to interact with PEBP2. Also attachment of the region aa 1–137 to the same region (aa 152–419) in the construct del 138–152 conferred the ability to interact with PEBP2. Therefore, we conclude that the region aa 67–151 of MITF mediates the physical interaction, and is responsible for the synergistic transactivation with PEBP2, and that this region may be subdivided into subregions.

In melanocytes, the transcriptional coactivators CBP/p300 selectively associate with the MITF that is phosphorylated at Ser^{77} by the mitogen-activated protein kinase (MAPK), and coactivate the transcriptional function of MITF (35, 36). Sato et al. reported that the region aa 123–127, overlapping with the PEBP2 interaction region, was important for the interaction with CBP/p300 (37). In the present study, however, MITF showed full and partial activities of the synergistic transactivation with PEBP2 even when the MAPK phosphorylation site and the CBP/p300 interaction site of MITF were deleted, respectively. There is the possibility that the phosphorylation and the subsequent association with CBP/p300 might not be essential at least for the synergistic transactivation of MMCP-6 promoter by MITF and PEBP2.

Other MITF-interacting transcription factors, TFE3, TFEB and TFEC, all of which belong to the bHLH-Zip family, interact through the HLH-Zip domains (3). Therefore, the interaction in the N-terminal region of MITF revealed in the present study may be a unique feature of the MITF-PEBP2 interaction.

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