Interaction and Cooperation of mi Transcription Factor (MITF) and Myc-associated Zinc-finger Protein-related Factor (MAZR) for Transcription of Mouse Mast Cell Protease 6 Gene*

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Eiichi Morii‡§, Keisuke Oboki‡, Tatsuki R. Kataoka‡, Kazuhiko Igarashi‡, and Yukihiko Kitamura‡

From the ‡Department of Pathology (Room C2), Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka, 565-0871, Japan, and the §Department of Biochemistry, Hiroshima University Medical School, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

The mi transcription factor (MITF) is a basic-helix-loop-helix leucine zipper (bHLH-Zip) transcription factor that is important for the normal phenotypic expression of mast cells. Most transcription factors function in cooperation with other factors by protein-protein interactions. To search proteins interacting with MITF, we carried out a yeast two-hybrid screen and isolated Myc-associated zinc-finger protein related factor (MAZR) as a partner of MITF. When expressed with MITF in NIH/3T3 cells, MAZR was colocalized with MITF. The association of MAZR with MITF was further confirmed by a co-immunoprecipitation study and in vitro binding assay. The zinc-finger domain of MAZR and the Zip domain of MITF were essential for the interaction. MAZR was expressed in cultured mast cells and MST mastocytoma cells containing mouse mast cell protease (mMCP)-6 transcript abundantly. The overexpression of dominant negative MAZR in MST mastocytoma cells reduced the amount of mMCP-6 mRNA. The simultaneous transfection of MAZR and MITF significantly increased the promoter activity of the mMCP-6 gene, indicating that the MAZR and MITF synergistically transactivated the mMCP-6 gene. MAZR appeared to play important roles in the normal phenotypic expression of mast cells in association with MITF.

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 MITF) (1, 2). The MITF encoded by the mutant mi allele (mi-MITF) deletes one of four consecutive arginines in the basic domain (1, 3, 4). The mi/mi mutant mice show microphthalmia, depletion of pigmentation in both hair and eyes, osteopetrosis, and deficient natural killer activity (3, 5–7). In addition, the phenotypic expression of mast cells in association with MITF.

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Among the genes whose expression was deficient in mi/mi CMCs, the transactivation mechanism of the mMCP-6 gene has been studied most intensively (18, 25, 26). The expression of mMCP-6 gene was deficient not only in mi/mi CMCs but also in CMCs derived from other mutants at the mi locus. The tg/tg CMCs that lack MITF did not express the mMCP-6 gene (27). The mi/mi/mi/mi and mi/mi/mi/mi CMCs, in which the basic domain and the Zip domain of MITF were deleted, respectively, also did not express the mMCP-6 gene (28, 29). These findings indicated that the normal (+) MITF was essential for the expression of the mMCP-6 gene. The +/−MITF bound the three motifs in the promoter region of the mMCP-6 gene (18). The mutation of each of these three motifs reduced the magnitude of transactivation by +/−MITF. Among them, the GACGG motif appeared to play the most important role since the magnitude of reduction was greatest after the mutation of the GACCTG motif (18). We found that the GACCTG motif was partly overlapped by the binding motif recognized by another transcription factor, polyomavirus enhancer-binding protein (PEBP2). PEBP2 interacted with +/−MITF and showed functional synergy in the transcription of mMCP-6 gene (26).

In the present study, we searched the factor that cooperated with +/−MITF using the yeast two-hybrid screen and found the Myc-associated zinc-finger protein-related factor (MAZR) as a protein that interacted with +/−MITF. MAZR possesses the broad-complex-tramtrack-bric-a-brac (BTB) domain and the zinc-finger domain (30). The overexpression of dominant negative MAZR reduced the expression of the mMCP-6 gene in mastocytoma cells. MAZR showed functional synergy with +/−MITF for transcription of the mMCP-6 gene. MAZR appeared to play roles in the normal phenotype expression of mast cells in association with +/−MITF.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—The bait plasmid pGBKT7-MITF was constructed by inserting a portion of MITF cDNA, which was deleted in the N-terminal 161 residues, into pGBKT7 vector (CLONTECH, Palo Alto, CA). The yeast two-hybrid screen was performed according to the instructions for the MATCHMAKER two-hybrid system 3 (CLONTECH) using pGBKTT-MITF as bait and a mouse lymphoma cell cDNA library (CLONTECH). Approximately 2 × 10^6 Hf7c yeast transformants were screened for His autotrophy. β-galactosidase assay was used for isolating positive clones. The isolated clones were sequenced in both directions.

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‡ To whom correspondence should be addressed. Tel.: 81-6-6879-3721; Fax: 81-6-6879-3729, E-mail: morii@patho.med.osaka-u.ac.jp.

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¶ The abbreviations used are: bHLH-Zip, basic-helix-loop-helix leucine zipper; MITF, mi transcription factor; CMC, cultured mast cells; MST, mastocytoma cell line; PEBP, polyomavirus enhancer-binding protein; MAZR, Myc-associated zinc-finger protein-related factor; BTB, broad-complex-tramtrack-bric-a-brac; GST, glutathione S-transferase; GFP, green fluorescent protein; Ab, antibody(ies); nt, nucleotide(s); aa, amino acid(s).
tions with ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

**Cells**—CMCs were obtained by culturing spleen cells of +/+ mice as described previously (17). MST cells were kindly provided by Dr. J. D. Eako of University of California, San Diego (21), and Jurkat cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (Nippon Bio Supply Center, Tokyo, Japan). NIH/3T3 cells and 293T cells were maintained in Dulbecco’s modification of Eagle’s medium (Flow Laboratories, Irvine, UK) supplemented with 10% fetal calf serum.

**Plasmids**—The DNA fragment encoding the entire open reading frame of MAZR was obtained by PCR from the reverse-transcribed product of CMCs. The used primers were 5′-ATGGAGCCGCTCAACGGCAGCTCTTTGGCGGT and 5′-TCATCTCCCTCGGACCCATGCTG-GCTG. The amplified DNA fragment was subcloned into pBluescript (Stratagene, La Jolla, CA). The amplified fragment was also subcloned into pGEX-3X glutathione S-transferase (GST)-expressing vector (Amersham Biosciences, Inc.) and into pEF-BOS expression vector kindly provided by Dr. S. Nagata of Osaka University (32). Various fragments of MAZR were amplified by PCR and subcloned into pBluescript or pEF-BOS. The expression plasmid containing MAZR fused with green fluorescent protein (GFP) and the expression plasmid containing MAZR fused with FLAG epitope tag were described before (30). The expression plasmid containing Myc-tagged normal or mutant MITFs, the expression plasmid containing Myc-tagged normal or mutant MITFs, and the GST-expressing plasmid containing +MITF were also described previously (19, 33, 34). All of the PCR fragments were verified by sequencing.

**Northern Blot Analysis**—Each RNA sample was prepared from 1.0 × 10^7 CMCs, MST cells, and Jurkat cells by the lithium chloride-urea method (35). Northern blot analysis was performed using the full-length MAZR (30), mMCP-6 (36), and glyceraldehyde-3-phosphate dehydrogenase (37) cDNAs labeled with [32P]dCTP (PerkinElmer Life Sciences; 10 mCi/ml) by random oligonucleotide priming. After hybridization at 42 °C, blots were washed to a final stringency of 0.2 × SSC (1 × SSC is 150 mmol/liter NaCl and 15 mmol/liter trisodium citrate, pH 7.4) and subjected to autoradiography.

**Immunocytochemistry**—The expression plasmid containing +MITF and the expression plasmid containing MAZR fused with GFP were cotransfected to NIH/3T3 cells using TransFast Transfection Reagent (Promega, Madison, WI) according to the manufacturer’s instructions. The cells were fixed with 100% methanol and permeated by treatment with 0.2% Triton X-100 in phosphate-buffered saline. The cells were incubated with polyclonal rabbit anti-MITF antibody (Ab). Immunoreactive cells were detected with anti-rabbit IgG Ab conjugated with rhodamine (MBL, Nagoya, Japan). The cells expressing +MITF were detected with the red filter, and the cells expressing MAZR fused with GFP were detected with the green filter. The specimens were observed with a confocal laser scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

**Immunoprecipitation**—The Myc-tagged normal or mutant MITF was coexpressed with FLAG-tagged MAZR in 293T cells using TransFast Transfection Reagent (Promega). The nuclear or whole cell extract was obtained by the method described previously (38). The nuclear or whole cell extract was incubated with LIP buffer (10 mM HEPES, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and protein G-Sepharose (Amersham Biosciences, Inc.) for 1 h with gentle rocking and centrifuged at 3,000 rpm for 3 min. The supernatants were transferred into new tubes and incubated with protein G-Sepharose and anti-Myc monoclonal Ab (9E10, PharMingen, San Diego, CA) or anti-FLAG Ab (Sigma) for 1 h in LIP buffer. Immunocomplexes were washed four times with LIP buffer, resuspended in loading buffer, boiled, and analyzed by immunoblot with anti-Myc monoclonal Ab.

**In Vitro Binding Assay**—The 32P-labeled normal or mutant MITF protein was synthesized using the reticulocyte lysate system (TNT system, Promega). The 32P-labeled normal or mutant MAZR protein was also synthesized. For the binding assays, the 32P-labeled normal or mutant MITF protein was incubated for 1 h at room temperature with GST-MAZR or GST alone immobilized on glutathione-agarose beads. The GST-MAZR or GST alone immobilized on beads were washed four times. Proteins retained on the beads were subsequently analyzed by SDS-PAGE and autoradiography.

**Transfection and Luciferase Assay**—The transfection to MST cells and to Jurkat cells were performed by electroporation. The reporter plasmid that contained the promoter region of the mMCP-6 gene starting from nucleotide (nt) −171 or nt −151 and the reporter plasmid with the minimal mMCP-6 promoter starting from nt −61 were described previously (26). The reporter plasmid mutated at the MITF-binding GACCTG motif in the mMCP-6 promoter and the reporter plasmid possessing the tetramer fragment between nt −171 and −151 upstream from the minimal mMCP-6 promoter were also described previously (26). The pEF-BOS expression plasmid containing +MITF, mutant MITFs, MAZR, or mutant MAZRs was used as the effector plasmid. In luciferase assays, 5 μg of a reporter, 2 μg of effector plasmids, and 1 μg of an expression vector containing the β-galactosidase gene were cotransfected to Jurkat cells. The expression vector containing the β-galactosidase gene was used as an internal control. When two kinds of effector plasmids were used, equal amounts of both plasmids were transfected. In some experiments, 5 μg of a reporter, 2 μg of the expression plasmid containing +MITF, 2 or 4 μg of the expression plasmid containing the dominant negative form of MAZR and 1 μg of an expression vector containing the β-galactosidase gene were cotransfected to MST cells. The cells were harvested 48 h after the transfection and lysed with 0.1 mol/liter potassium phosphate buffer (pH 7.4) containing 1% Triton X-100. Soluble extracts were then assayed for luciferase activity with a luminometer LB966P (Berthold GmbH, Wildbad, Germany) and for β-galactosidase activity. The luciferase activity was normalized by the β-galactosidase activity and the total protein concentration as described previously (18). The normalized value was divided by the value obtained without effector plasmids, and the divided value was expressed as the relative luciferase activity.

**RESULTS**

We searched for proteins that were associated with +MITF by yeast two-hybrid screening. The full-length +MITF was not suitable as bait, since the full-length +MITF fused to the Gal4 DNA binding domain was a strong transactivator of reporter genes. We deleted the transactivation domain and obtained the new construct, truncating the N-terminal 161 amino acids (aa) of MITF (MITF(162−419)). MITF(162−419) contained the bHLH-Zip domain. MITF(162−419) fused with the Gal4 DNA binding domain was used as bait. Positive transformants were selected for His-autotrophy and β-galactosidase assay. We isolated eighteen positive cDNA clones. Four of the 18 clones encoded the ubiquitin-conjugating enzyme UBC9, and one of the 18 clones encoded protein kinase C-interacting protein. UBC9 and protein kinase C-interacting protein were previously shown to associate with MITF (39, 40). One of the other clones was found to encode a part of cDNA of a transcription factor, MAZR. MAZR consisted of 641 aa and contains a BTB domain in the N terminus (aa 1–145) and seven zine-finger domains in the C terminus (aa 288–641) (30). By Northern blotting, the expression of the MAZR gene was detected in CMCs and MST mastocytoma cells, but was hardly detectable in Jurkat T cells (Fig. 1A).

The interaction of MAZR to +MITF was confirmed by two experiments. First, MAZR fused with GFP was coexpressed with +MITF in NIH/3T3 cells, and their subcellular localization was examined. The +MITF and MAZR were colocalized in the nucleus of NIH/3T3 cells (Fig. 2A). Next, the co-immunoprecipitation studies were performed. Myc-tagged +MITF and FLAG-tagged MAZR were coexpressed in 293T cells, and their nuclear extract was analyzed. The immuno-
precipitated product with anti-FLAG Ab contained Myc-tagged +MITF (Fig. 2B).

To identify the region of +MITF that was required for the interaction with MAZR, we carried out in vitro binding experiments. Various mutants of MITF were constructed (Fig. 3A). The mi-MITF deletes one arginine in the basic domain. MITF-(1–260) deletes the Zip region downstream of the Zip domain. MITF-(1–260) deletes the Zip domain. The 35S-labeled normal or mutant MITF was subjected to coprecipitation with GST or GST-MAZR fusion protein that was immobilized on glutathione-agarose beads. Protein complexes were analyzed by SDS-PAGE. The complex of +MITF and GST-MAZR was detected, but that of +MITF and GST was not (Fig. 3B). The mi-MITF and MITF-(1–298) bound GST-MAZR-coated beads but not GST-coated beads (Fig. 3B). In contrast, MITF-(1–260), which lacked the Zip domain, did not bind GST-MAZR-coated beads (Fig. 3B).

The region required for the interaction with MAZR was also examined by a co-immunoprecipitation study. Myc-tagged +MITF, Myc-tagged mi-MITF, and Myc-tagged MITF-(1–260) were coexpressed with FLAG-tagged MAZR in 293T cells, and their whole cell lysate was analyzed. The immunoprecipitated product with anti-FLAG Ab contained Myc-tagged +MITF or Myc-tagged mi-MITF, but did not contain Myc-tagged MITF-(1–260) that lacked the Zip domain (Fig. 3C).

Next, the region of MAZR necessary for the interaction with MITF was examined by an in vitro binding experiment. MAZR deleting the zinc-finger domain (MAZR-(1–288)), MAZR deleting the BTB domain (MAZR-(145–641)), and MAZR containing the zinc-finger domain alone (MAZR-(288–641) were constructed (Fig. 4A). MAZR, MAZR-(145–641), and MAZR-(288–641) bound GST- +MITF-coated beads but not GST-coated beads (Fig. 4B). In contrast, MAZR-(1–288) that lacked the zinc-finger domain did not bind GST- +MITF-coated beads (Fig. 4B).

Since the +MITF strongly transactivated the mMCP-6 promoter in mast cells (18), the effect of MAZR on the function of +MITF was examined. To examine whether the endogenous MAZR increased the amount of mMCP-6 mRNA, we used a dominant negative MAZR. The dominant negative MAZR is a mutant that possesses only a part of the zinc-finger domain (aa 409–496). We overexpressed the dominant negative MAZR in MST cells that expressed both mMCP-6 and MAZR mRNAs. After 6 days of culture, the expression of mMCP-6 gene was examined by Northern blotting. The overexpression of dominant negative MAZR significantly reduced the amount of mMCP-6 mRNA (Fig. 5A). Next, we cotransfected the dominant negative MAZR into MST cells with the reporter plasmid containing the promoter region of the mMCP-6 gene. This reporter plasmid possessed the GACCTG motif to which +MITF bound. When various amounts of dominant negative MAZR were cotransfected, the luciferase activity of the reporter plasmid decreased in a dose-dependent manner (Fig. 5B).

The functional synergy between +MITF and MAZR was examined. To remove the possible involvement of endogenous MITF and MAZR, we used Jurkat cells that expressed neither MITF (19) nor MAZR. The expression plasmid containing +MITF cDNA and that containing MAZR cDNA were cotransfected to Jurkat cells with the reporter plasmid used in Fig. 5B. The transfection of the plasmid containing +MITF cDNA alone and the transfection of the plasmid containing MAZR
The synergy with MAZR was not observed when mi-gene abolished the synergy (Fig. 8). The simultaneous transfection of these plasmids increased the luciferase activity. In contrast, cDNA alone did not increase the luciferase activity. In contrast, the simultaneous transfection of these plasmids increased the luciferase activity ~40-fold (Fig. 6).

Various mutants of MITF were cotransfected with MAZR. The synergy with MAZR was not observed when mi-MITF or MITF-(1–260) deleting the Zip domain was cotransfected (Fig. 7). Next, various mutants of MAZR were cotransfected with +MITF. MAZR-(145–641) and MAZR-(288–641) showed the synergy with +MITF, but MAZR-(1–288) deleting the zinc-finger domain did not (Fig. 7).

Then, we examined the DNA element that was necessary for the synergy between MAZR and +MITF. The +MITF bound the GACCTG motif in the mMCP-6 promoter but not the mutated GCCTG motif (Fig. 8A). The mutation or deletion of the GACCTG motif abolished the synergy (Fig. 8B). The tetrameric fragments between nt −171 and −151 were cloned into the plasmid with the minimal mMCP-6 promoter starting from nt −61. A significant synergy between +MITF and MAZR was observed in the reporter plasmid containing the tetrameric fragments but not in the reporter plasmid containing the minimal mMCP-6 promoter alone (Fig. 8C). No synergy was observed in the reporter plasmid that contained the tetrameric fragments mutated at the GACCTG motif (Fig. 8C).

**DISCUSSION**

MAZR was isolated as a protein that interacted with +MITF by yeast two-hybrid screening. MAZR was first cloned as the protein interacting with Bach2, which is a transcription factor possessing both the BTB domain and the b-Zip domain (30). MAZR transactivates the c-myc gene in B cells and is important for the development of B cells in association with Bach2 (30). In mast cells, MAZR transactivated the mMCP-6 gene in cooperation with +MITF. MAZR may show synergy with +MITF in the transcription of other genes in mast cells. MAZR appeared to play some roles in the normal phenotype expression of mast cells in association with +MITF.

The deletion of the zinc-finger domain of MAZR abolished the physical interaction and functional synergy with +MITF. This suggested that the zinc-finger domain of MAZR was essential for the interaction with +MITF.

The Zip domain of MITF mediated the physical interaction and functional synergy with MAZR. The Zip domain of cAMP-response element-binding protein interacted with the zinc-finger domain of YY1 (41). The present result may be another example of such interactions.

The MITF encoded by the mutant mit^ allele deletes the Zip domain (3). The mMCP-6 gene was not expressed in mast cells of mit^/mit^ genotype (28). Since the Zip domain of MITF mediated the interaction with MAZR, the loss of expression of the mMCP-6 gene might be attributable to the abolishment of
synergy between MITF and MAZR in m<sup> mice/m<sup> mice</sup> mast cells. The MITF encoded by the mutant m<sup> mice/MITF</sup> allele (m<sup> mice-MITF</sup>) deletes most of the portion of the basic domain (3). The mMCP-6 gene was also not expressed in mast cells of the m<sup> mice/m<sup> mice</sup> genotype (29). Since the basic domain of MITF mediates the DNA binding (4), the loss of expression of the mMCP-6 gene might be attributable to the deficient DNA binding of the complex of m<sup> mice-MITF</sup> and MAZR in m<sup> mice/m<sup> mice</sup> mast cells.

MAZR recognizes the G-rich motif of DNA (30). The consensus binding sequence of MAZR is G(C)/GGG(GGG/GA/C/C (30). In the promoter region of the mMCP-6 gene, there was a sequence GTGGG(GGG/GA/C/C between nt -138 and -128, in which nine of 11 nucleotides were matched to the MAZR-consensus sequence. However, this sequence was not essential for the synergy between +MITF and MAZR, since the synergy was observed in the reporter plasmid containing the fragment between nt -171 and -151 alone upstream of the minimal mMCP-6 promoter. No G-rich motif was found in the fragment between nt -171 and -151 or in the minimal mMCP-6 promoter. The G-rich motif appeared dispensable for the synergy between MAZR and +MITF. Instead, the GACCTG motif between nt -166 and -161, to which +MITF bound, was required for the synergy, since the mutation at the GACCTG motif abolished it. The binding of +MITF to the GACCTG motif activated the mMCP-6 promoter, and binding of MAZR to +MITF might further enhance the promoter activity.

PEBP2 showed synergy with +MITF for the transcription of the mMCP-6 gene (26). PEBP2 recognized the TGTTGTCG motif, partly overlapped the MITF-binding GACCTG motif (the overlapped nucleotides were underlined) (26). The +MITF interacted with PEBP2 through the region upstream of the basic domain (33), whereas the +MITF interacted with MAZR through the Zip domain. The formation of the triple complex consisting of +MITF, PEBP2, and MAZR might be possible. The triple complex might efficiently enhance the transcription of the mMCP-6 gene in mast cells.

Taken together, MAZR interacted with +MITF and synergistically transactivated the mMCP-6 promoter. MAZR appeared important for the normal phenotypic expression of mast cells.

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REFERENCES


Fig. 7. Synergy between various mutant MITFs and mutant MAZRs. Various mutant MITFs and MAZR were coexpressed in Jurkat cells with the reporter plasmid containing the mMCP-6 promoter used in Figs. 5 and 6. In separate transfections, the +MITF and various mutant MAZRs were coexpressed. The transduced DNA was always kept in equal amounts using the backbone expression vector in each transfection assay. The values represent the means ± S.E. of three experiments. In some cases, the S.E. was too small to be shown by bars. *, p < 0.01 by t test when compared with the luciferase activity obtained by the transfection of the reporter plasmid without effector plasmids.

Fig. 8. Synergy between +MITF and MAZR in various reporter plasmids. A, the sequence of mMCP-6 promoter between nt -171 and -151. The MITF-binding GACCTG motif is boxed. The mutated GACCTG motif is also shown. B, the reporter plasmid containing the GACCTG motif or the reporter plasmid mutating or deleting the GACCTG motif was cotransfected with the effector plasmids containing +MITF and MAZR. C, the reporter plasmid containing the tetrameric fragments between nt -171 and -151 upstream of the minimal mMCP-6 promoter was cotransfected with the effector plasmids. The reporter plasmid with the tetrameric fragments mutated in the GACCTG motif was also used. The values represent the means ± S.E. of three experiments. In some cases, the S.E. was too small to be shown by bars. *, p < 0.01 by t test when compared with the luciferase activity obtained by the transfection of the reporter plasmid without effector plasmids.
Synergy of MITF and MAZR

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