Regulation of Mouse Mast Cell Protease 6 Gene Expression by Transcription Factor Encoded by the mi Locus

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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). Because the expression of the mouse mast cell protease 6 (MMCP-6) gene is remarkably reduced in mast cells of mi/hi mutant mice, we investigated the effect of MITF on the transcription of the MMCP-6 gene. First, we introduced the normal (+) MITF cDNA into mi/hi cultured mast cells using the retroviral vector. Overexpression of + MITF but not mi-MITF normalized the expression of the MMCP-6 gene, indicating the involvement of + MITF in the MMCP-6 gene transcription. Second, we analyzed the promoter of the MMCP-6 gene by the transient cotransfection assay. The luciferase construct under the control of the MMCP-6 promoter and the cDNA encoding + MITF or mi-MITF were cotransfected into NIH/3T3 fibroblasts. The coexpression of + MITF but not mi-MITF increased the luciferase activity 10-fold. We found a CACATG and a CATCTG motif in the MMCP-6 promoter, both of which are generally recognized by bHLH-Zip-type transcription factors. We also found a GACCTG motif that was strongly bound by + MITF. These three motifs were necessary for the 10-fold transactivation ability of the MMCP-6 promoter by + MITF. Mutations of each motif significantly reduced the transactivation, suggesting that + MITF directly transactivated the MMCP-6 gene through these three motifs.

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MATERIALS AND METHODS

Mice. The original stock of C57BL/6-mi/+ (mi/+ ) mice was purchased from the Jackson Laboratory (Bar Harbor, ME) and were maintained in our laboratory by consecutive backcrosses to our own inbred C57BL/6 colony (more than 12 generations at the time of the present experiment). Female mi/+ mice were crossed with male mi/+ mice, and the resulting mi/hi mice were selected by their white coat color. The mi/hi mice were crossed with male C57BL/6 mice, and the resulting mi/hi mice were selected by their white coat color.

Cells. Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al.32 Mice of mi/hi genotype and their normal (+/+ ) littermates were used at 2 to 3 weeks of age to obtain CMCs. Mice were killed by decapitation after ether anesthesia and spleens were removed. Spleen cells derived from mi/hi or +/+ mice were cultured with α-minimal essential medium (α-MEM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10% PWM-SCM and 10% fetal calf serum (FCS; Nippon Bio-supp Center, Tokyo, Japan). Half of the medium was replaced every 7 days. For the morphological examination, CMCs were collected, washed with phosphate-buffered saline (PBS), and cytocentrifuged. The specimens were fixed with Carnoy’s solution (60% ethanol, 30% chloroform, and 10% acetic acid) for 30 minutes and then stained with Alcian blue and nuclear fast red.

More than 95% of cells were CMCs 4 weeks after the initiation of the culture. A helper virus-free packaging cell line (φ2) was maintained in Dulbecco’s modified Eagle’s medium (DMEM; ICN Biomedicals) supplemented with 10% FCS. The NIH/3T3 fibroblast

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cell line was generously provided by Dr S.A. Aaronson (National Cancer Institute, Bethesda, MD) and maintained in DMEM supplemented with 10% FCS.

Construction of retrovirus and its infection. Bluescript KS(−) plasmids (pBS; Stratagene, La Jolla, CA) containing the whole coding regions of +/MITF or mi-MITF (pBS- +MITF and pBS-mi–MITF, respectively) had been constructed in our laboratory.18 A retroviral vector pM5Gneo,23 a derivative of myeloproliferative sarcoma virus vector, was a kind gift from Dr W. Osteregg (Universität Hamburg, Hamburg, Germany). The purified Sma I-HincII fragment from pBS- +MITF or pBS-mi–MITF was introduced into the blunted EcoRI site of pM5Gneo. The resulting plasmids were transfected into packaging cell line (2/2)26 by calcium phosphate method,14 and neomycin-resistant 2/2 cell clones were selected by culturing in DMEM containing 10% FCS and G418 (0.8 mg/mL; GIBCO-BRL, Grand Island, NY). For the gene transfer, spleen cells obtained from mifmi mice were incubated on irradiated (30 Gy) subconfluent monolayer of virus-producing 2/2 cells for 72 hours in α-MEM supplemented with 10% PWM-SCM and 10% FCS. Neomycin-resistant CMCs were obtained by culturing the culture in α-MEM containing 10% PWM-SCM, 10% FCS, and G418 (0.8 mg/mL) for 4 weeks.

Northern blot analysis. Total cell RNAs were prepared by lithium chloride-urea method.29 The fragment of +/MITF (nt 433 to 1,176),30 MMCP-6,31 mouse mast cell carboxypeptidase A (MC-CPA),29 and β-actin27 cDNAs were used as probes after being labeled with [32P]-α–dCTP (DuPont/NEN Research Products, Boston, MA; 100 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/L NaCl, 15 mmol/L trisodium citrate, pH 7.4) at 50°C and subjected to autoradiography.

Preparation of anti-MITF antibody (AB) and immunocytochemistry. A peptide corresponding to C-terminal of MITF (amino acids 404 to 419; SRRR SSSA ETEHAC) was synthesized and conjugated with bovine serum albumin (BSA). The conjugate (500 μg) was subcutaneously injected to a rabbit every 3 weeks. The serum was obtained 6 weeks after the first immunization and purified by resin coupled with the synthetic peptide. The manuscript describing the specificity of this AB has been submitted elsewhere (K. Takebayashi et al).

Cells were collected, washed with PBS, fixed with 4% paraformaldehyde in 0.1 mol/L PB overnight, and embedded in paraffin. A microwave treatment (H2590 Microwave Processor; Bio-Rad Laboratories, Hercules, CA) in 0.01 mol/L citrate buffer (pH 6.0) for 10 minutes, sections (4 μm) were incubated with 0.3% H2O2 in methanol for 30 minutes. After washing with PBS, the sections were incubated with the anti-MITF AB (1 μg/mL; in PBS containing 0.1% BSA and 0.1% Tween 20) at 4°C overnight. The sections were washed with PBS for 5 minutes and then incubated with the biotin-conjugated goat antirabbit IgG AB (DAKO A/S, Glostrup, Denmark). Immuno-reacted cells were visualized with streptavidin-biotin-peroxidase and 0.05% diaminobenzidine-0.02% H2O2 solution (DAKO A/S) according to the manufacturer’s instructions.

In situ hybridization. Cells were collected, washed with PBS, fixed with 4% paraformaldehyde in 0.1 mol/L PB (pH 7.4) overnight, and embedded in paraffin. A mixture of MMP-6 cDNA and MC-CPA probes and the technique of in situ hybridization were described previously.28 The number of MMCP-6 mRNA+ cells and that of Akt/mice + cells were counted in the serial sections, and the proportion of MMCP-6 mRNA+ cells was calculated. The proportion of MC-CPA mRNA+ cells was also calculated.

Construction of effector and reporter plasmids. pEF-BOS expression vector was kindly provided by Dr S. Nagata (Osaka Bioscience Inst., Osaka).29 The Sma I-HincII fragment of pBS- +MITF or pBS-mi–MITF was introduced into blunted Xba I site of pEF-BOS. The luciferase gene subcloned into pSP72 (pSPLuc) was generously provided by Dr K. Nakajima (Osaka University Medical School, Osaka).30 To construct reporter plasmids, a DNA fragment containing a promoter region and the first exon (noncoding region) of the MMCP-6 gene (~191 to +26) was cloned into the upstream of luciferase gene in pSPLuc. The MMCP-6 promoter deletions were produced by PCR using the appropriate 5′ primers and a common 3′ primer located between +6 and +26. The mutations were introduced by PCR with mismatch primers. Deleted or mutated products were verified by sequencing.

Transient cotransfection assay. NIH/3T3 cells (5 × 104) were plated in a 10 cm dish 1 day before the procedure. Cotransfection of 10 μg of a reporter, 5 μg of an effector and 5 μg of an expression vector containing β-galactosidase gene was performed by the calcium phosphate precipitation method.29 The expression vector containing β-galactosidase gene was used as an internal control. The cells were obtained 48 hours after the transfection and lysed with 0.1 mol/L potassium phosphate buffer (pH 7.4) containing 1% Triton X-100 (Sigma). 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 by the β-galactosidase activity and total protein concentration according to the method described by Yasumoto et al.31 The normalized value was divided by the value obtained with the cotransfection of the reporter and pEF-BOS, and was expressed as the relative luciferase activity. EGMSA. The production and purification of glutathione-S-transferase (GST)–+MITF and GST–mi-MITF fusion proteins were described previously.32 Oligonucleotides were labeled with α-[32P]-dCTP by filling 5′-overhangs and used as probes of EGMSA. DNA-binding assays were performed in a 20 μL reaction mixture containing 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 75 mmol/L KCl, 1 mmol/L dithiothreitol, 4% Ficoll type 400, 50 ng of poly (dl-dc), 25 ng of labeled DNA probe, and 3.5 ng of GST-+MITF or GST-mi-MITF fusion proteins. After the incubation at room temperature for 15 minutes, the reaction mixture was subjected to electrophoresis at 14 V/cm at 4°C on a 5% polyacrylamide gel in 0.25 × TBE buffer (1 × TBE is 90 mmol/L Tris-HCl, 64.6 mmol/L boric acid, and 2.5 mmol/L EDTA, pH 8.3). The polyacrylamide gels were dried on Whatman 3MM chromatography paper and subjected to autoradiography.

RESULTS

Introduction of +/MITF cDNA into mifmi CMCs. Spleen cells of mifmi mice were incubated for 2 days on 2/2 cells that produced viruses containing +/MITF or mi-MITF cDNA. The neomycin-resistant cells were selected by culturing for 4 weeks in α-MEM containing G418 and PWM-SCM. More than 95% of the surviving cells contained Alcian blue+ granules, as in the case of mifmi CMCs that were established in the absence of 2/2 cells. Although the mRNA expression of endogenous mi-MITF was hardly detectable, the expression of introduced +/MITF or mi-MITF cDNA was apparent (Fig 1A). Moreover, content of +/MITF in the nucleus and that of mi-MITF in the cytoplasm was demonstrable using the anti-MITF AB (Fig 1B).

The MMCP-6 mRNA expression was examined in +/+ CMCs, mifmi CMCs, mifmi CMCs overexpressing +/MITF and mifmi CMCs overexpressing mi-MITF. As reported previously,29 +/+ CMCs strongly expressed the MMCP-6 mRNA, whereas the expression in mifmi CMCs was hardly detectable. The expression of MMCP-6 mRNA recovered to the nearly normal level in mifmi CMCs overexpressing +/MITF.

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MITF but not in mi/mi CMCs overexpressing mi-MITF. The introduction of +/+-MITF or mi-MITF cDNA did not affect the expression of MC-CPA mRNA at all (Fig 1C).

The expression of MMCP-6 mRNA in +/+ CMCs, mi/mi CMCs, mi/mi CMCs overexpressing +/+-MITF and mi/mi CMCs overexpressing mi-MITF was also examined by in situ hybridization. Approximately a half of +/+ CMCs expressed MMCP-6 mRNA, whereas very few mi/mi CMCs expressed it (Table 1). The introduction of +/+-MITF cDNA but not mi-MITF cDNA significantly increased the proportion of MMCP-6 mRNA-expressing cells. The proportion of MC-CPA mRNA expressing cells was not affected by the introduction of mi-MITF or +/+-MITF cDNA into mi/mi CMCs.

Transactivation effect of MITF on the MMCP-6 promoter.
We attempted to identify the motifs that mediated the transactivation effect of MITF. In the previous report,18 we compared the promoter region of the MMCP-6 gene with that of the HSMCT gene, and found a highly conserved region containing a CACATG motif (nt -128 to -123). We showed the binding of +/+-MITF to the CACATG motif.15 In the present study, we examined whether the CACATG motif practically mediated the transactivation effect of MITF. The promoter region and the first exon of MMCP-6 gene (nt -191 to +26) was cloned upstream of the luciferase gene. The expression plasmid containing +/+-MITF or mi-MITF cDNA was cotransfected into NIH/3T3 fibroblasts with the luciferase construct containing the MMCP-6 promoter. The coexpression of +/+-MITF increased the luciferase activity approximately 10-fold (Fig 2). In contrast, the coexpression of mi-MITF did not increase the luciferase activity. Furthermore, we cotransfected the luciferase construct with both +/+-MITF cDNA and an excess amount of mi-MITF cDNA. The increased luciferase activity induced by +/+-MITF was not affected by the excess amount of mi-MITF cDNA (data not shown).

Table 1. Increase of MMCP-6-mRNA Expressing Cells by Introduction of +/+-MITF cDNA in mi/mi CMCs

<table>
<thead>
<tr>
<th>Type of Cells</th>
<th>Proportion of MMCP-6 mRNA to MC-CPA mRNA (%)</th>
<th>Proportion of Alcian Blue Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+ CMCs</td>
<td>54 ± 3.1</td>
<td>90 ± 1</td>
</tr>
<tr>
<td>mi/mi CMCs</td>
<td>1 ± 2</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>mi/mi CMCs overexpressing +/+-MITF</td>
<td>32 ± 2.1</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>mi/mi CMCs overexpressing mi-MITF</td>
<td>1 ± 1</td>
<td>90 ± 2</td>
</tr>
</tbody>
</table>

* Mean ± SE of three experiments.
† P < .01 by t-test when compared with the value of +/+ CMCs.
‡ P < .01 by t-test when compared with the value of +/+-MITF.
The deletion of the promoter sequence from nt -171 abolished the transactivation ability of +M-IF. No apparent homology with the promoter region of HSMCT was observed between nt -171 and -161, and no consensus sequences for any transcription factors were detectable. However, the sequence between nt -166 and -161, GACCTG, was similar to the CANTTG motif (Fig 3). When the oligonucleotide containing the GACCTG motif was used as a probe of EGMSA, strong binding of +M-IF was observed (Fig 4A). The excess amount of the oligonucleotide containing the GACCTG motif competed for the binding of +M-IF to the GACCTG motif. In contrast, the excess amount of oligonucleotide mutated in the GACCTG motif (ie, GTCCAG) did not compete (Fig 4A). These results indicated that +M-IF bound the GACCTG motif specifically. We produced the reporter plasmid which started from nt -191 containing the mutation in the GACCTG motif. The mutation decreased the magnitude of transactivation by M-IF from 10-fold to twofold (Fig 4B).

There was another CANNTG motif in the MMCP-6 promoter (Fig 3). The CATCTG motif between nt -145 and -140 was present just upstream of the CACATG motif (nt -128 to -123). This putative binding site of bHLH family transcription factors was not observed in the promoter region of the HSMCT gene (Fig 3). When the oligonucleotide with the CATCTG motif was used as a probe of EGMSA, the binding of +M-IF was hardly detectable (Fig 5A). However, the excess amount of the oligonucleotide with the CATCTG motif competed for the binding of +M-IF to the CATCTG motif (Fig 5B). The excess amount of the oligonucleotide mutated either in the CATCTG motif or in the CATCTG motif did not show any competition (Fig 5B). These results suggested the binding of +M-IF to the CATCTG motif with a low affinity. The mi-MIF bound neither the CACATG nor the CATCTG motif (Fig 5A). We produced the reporter plasmid in which the CATCTG motif was mutated to CCTCAG and the other reporter plasmid in which both the CACATG and CATCTG motifs were mutated. Both reporters started from nt -191. When +M-IF cDNA was cotransfected with the luciferase gene under the control of the normal MMCP-6 promoter, the magnitude of transactivation ability was 10-fold. The mutation at the CATCTG motif decreased the magnitude of transactivation from 10-fold to fivefold, and the mutation at both the CACATG and CATCTG motifs were mutated. Both reporters started from nt -191. When +M-IF cDNA was cotransfected with the luciferase gene under the control of the normal MMCP-6 promoter, the magnitude of transactivation ability was 10-fold. The mutation at the CATCTG motif decreased the magnitude of transactivation from 10-fold to fivefold, and the mutation at both the CACATG and CATCTG motifs completely abolished the transactivation ability of the +M-IF (Fig 5C).

**DISCUSSION**

The expression of the MMCP-6 gene was significantly lower in mi/mi CMCs than in +/- CMCs as described previously. The present result that the overexpression of +
MITF in mi/mi CMCs normalized the transcription of the MMCP-6 gene directly showed the involvement of +MIF in the transcription of the MMCP-6 gene. On the other hand, the overexpression of mi-MITF in mi/mi CMCs did not increase the expression of the MMCP-6 gene. We previously showed the loss of DNA binding ability of mi-MITF. A part of the defective function of mi-MITF may be attributable to its defective DNA binding ability. Furthermore, we have recently found a defective nuclear localization of mi-MITF. The mi-MITF was predominantly localized in the cytoplasm when it was transfected into NIH/3T3 fibroblasts. In the

**Fig 4.** The binding of +MITF to the GACCTG motif and the effect of coexpression of +MITF or mi-MITF cDNA on the luciferase activity under the control of the normal or mutated MMCP-6 promoter. (A) Competition for the binding of GST- +MITF. The labeled 5'-CCACCGTGTGACCTG&GGTCATCA oligonucleotide (oligo 1) was used as a probe (hexameric motif is shown by the underline and boxed by dotted lines in the figure). The excess amounts of nonlabeled oligo 1 or oligo 2 were added. In oligo 2, the GACCTG motif was mutated to GTCCAG (the mutated nucleotides are underlined). (B) The effect of the mutation at the GACCTG motif (to GTCCAG) on the luciferase activity. All MMCP-6 promoters started from nt−191. Bars indicate the SE of three assays.

**Fig 5.** The effect of coexpression of +MITF or mi-MITF cDNA on the luciferase activity under the control of the normal or mutated MMCP-6 promoter. (A) EMGS using GST- +MITF and GST-mi-MITF fusion proteins. The labeled 5'-TCAGAAGGCGTCTG&GGTGGTGGG oligonucleotide (oligo 3) and 5'-TGGTGGGACGCATGTTACATGGA oligonucleotide (oligo 5) were used as a probe (hexameric motifs are shown by underlines and boxed by solid lines in the figure). The sequences of the oligonucleotide mutated in the CATCTG motif (to CTTCAG, oligo 4) or in the CACATG motif (to CTCAAG, oligo 6) are also shown. The mutated nucleotides are underlined. (B) Competition for the binding of GST- +MITF to oligo 5. The excess amount of nonlabeled oligo 3, oligo 4, oligo 5, or oligo 6 was added. (C) The effect of the mutation at the CATCTG motif (to CTTCAG) and that of both the CATCTG (to CTTCAG) and CACATG (to CTCAAG) motifs on the luciferase activity. All MMCP-6 promoters started from nt−191. Bars indicate the SE of three assays.
present study, we examined the subcellular localization of +MITF and mi-MITF in CMCs using the anti-MITF AB. The +MITF was localized in the nucleus, whereas the mi-MITF was localized in the cytoplasm. This is consistent with the result obtained with NIH/3T3 fibroblasts. The loss of the nuclear localization potential may be another cause of the defective function of the mi-MITF in mast cells.

When the luciferase construct containing the MMCP-6 promoter was transfected into NIH/3T3 fibroblasts, the expression of +MITF but not mi-MITF increased the luciferase activity 10-fold. This indicated that the +MITF transactivated the luciferase gene through the MMCP-6 promoter. In the previous study, we found the consensus sequence, a CACATG motif, recognized by bHLH-Zip family of transcription factors by comparing the sequence of the promoter region between the MMCP-6 and HSMCT genes. In fact, the mutation at the CACATG motif reduced the transactivation effect from 10-fold to fourfold in the present study. This indicated that the CACATG motif played an important role for the transactivation induced by the +MITF.

The mutation at the CACATG motif reduced the transactivation activity, but it did not completely abolish it. This suggested the presence of other motifs that were necessary for the transactivation of the MMCP-6 gene. We produced the deleted reporter plasmids to examine the motifs mediating the transactivation ability besides the CACATG motif. The deletion from nt -171 completely abolished the transactivation induced by the +MITF. The result suggested that the element downstream from nt -171 was indispensable for the transactivation mediated by the +MITF. The sequence just downstream from nt -171 to nt -161 showed no apparent homology with the promoter region of HSMCT, and contained no consensus sequences for any known transcription factors. However, the GACCTG motif located between nt -165 and -161 was similar to the CANNTG motif recognized by bHLH-Zip family of transcription factors. The GACCTG motif was specifically bound by the +MITF, and the mutation in the GACCTG motif decreased the transactivation effect of the +MITF. These results suggested that the GACCTG motif mediated the transactivation of MMCP-6 promoter as well as CANNTG motifs.

We found another CANNTG consensus sequence, ie, the CATCTG motif just upstream of the previously found CACATG motif. Although the binding of +MITF to the CATCTG motif was hardly detectable with EGMSA, the excess amount of the oligonucleotides containing CATCTG motif competed for the binding of +MITF to the CATCTG motif. These results suggested that the +MITF may bind the CATCTG motif with a low affinity. In fact, the mutation at the CATCTG motif decreased the transactivation by the +MITF. The CATCTG motif appeared to play a significant role for the transactivation of the MMCP-6 gene. There is another possibility that MITF may not bind the CATCTG motif directly. MITF may activate factors that bind the CATCTG motif.

The expression of the MC-CPA gene did not decrease in mi/mi CMCs. The overexpression of +MITF did not affect the expression of the MC-CPA gene, either. Therefore, the +MITF does not appear to be involved in the transcription of the MC-CPA gene. Zon et al showed that other transcription factors, GATA-binding proteins, play an important role for the expression of the MC-CPA gene in mast cells. Because mast cell proteases are a group of major proteins in mast cell granules and because genes of various types of mast cell proteases have been cloned, they are a useful tool for studying the function of transcription factors in mast cells.

Taken together, the present results apparently showed the essential role of MITF for the expression of the MMCP-6 gene. The presence of the three motifs in the MMCP-6 promoter (GACCTG, CATCTG, and CACATG) was indispensable for the 10-fold transactivation by +MITF.

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