Number of Mast Cells in the Peritoneal Cavity of Mice

Influence of Microphthalmia Transcription Factor through Transcription of Newly Found Mast Cell Adhesion Molecule, Spermatogenic Immunoglobulin Superfamily

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The *mi* (microphthalmia) locus of mice encodes a transcription factor, MTF. B6-tg/tg mice that do not express any MTF have white coats and small eyes. Moreover, the number of mast cells decreased to one-third that of normal control (+/+) mice in the skin of B6-tg/tg mice. No mast cells were detectable in the stomach, mesentery, and peritoneal cavity of B6-tg/tg mice. Cultured mast cells derived from B6-tg/tg mice do not express a mast cell adhesion molecule, spermatogenic immunoglobulin superfamily (SgIGSF). To obtain *in vivo* evidence for the correlation of nonexpression of SgIGSF with decrease in mast cell number, we used another MTF mutant, B6-*mi*^{vit}/*mi*^{vit} mice that have a mild phenotype, ie, black coat with white patches and eyes of normal size. B6-*mi*^{vit}/*mi*^{vit} mice had a normal number of mast cells in the skin, stomach, and mesentery, but the number of peritoneal mast cells decreased to one-sixth that of +/+ mice. Cultured mast cells and peritoneal mast cells of B6-*mi*^{vit}/*mi*^{vit} mice showed a reduced but apparently detectable level of SgIGSF expression, demonstrating the parallelism between mast cell number and expression level of SgIGSF. The number of peritoneal mast cells appeared to be influenced by MTF through transcription of SgIGSF. (*Am J Pathol* 2004, 165:491–499)

(SCF) is the most important growth factor of mast cells and is encoded by the mouse *Sl* locus.^{1–4} KIT is the receptor for SCF and is encoded by the mouse *W* locus.^{1,5,6} MTF is a basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factor, and is encoded by the mouse *mi* locus.^{7–9} MTF regulates the expression of various genes in mast cells.^{10–18} The expression of KIT gene itself is impaired in mast cells of some MTF mutants.^{11,19–21}

Mast cells are mostly depleted in tissues of a severe KIT mutant, *W/W^v* mouse,⁵ and a severe SCF mutant, *Sl/Sl^d* mouse,² but the magnitude of mast cell deficiency was apparently milder in tissues of *W^v/W^v*, *W^d/W^d*, and *Sl^f/Sl^f* mutant mice, in which the abnormality of KIT or SCF was not so severe.^{22,23} Many mutants are known at the *mi* locus.^{24,25} When examined in C57BL/6 (B6) genetic background, the number of mast cells in the skin of B6-*mi/mi*, B6-*MI^{pr}/MI^{pr}*, B6-*mi^{ew}/mi^{ew}*, B6-*mi^{ce}/mi^{ce}*, and B6-*tg/tg* mice was one-third that of normal control (+/+) mice,^{19–21,26–28} but the number was normal in the skin of B6-*MI^{wh}/MI^{wh}* mice.¹⁹ The expression of KIT was deficient in cultured mast cells (CMCs) of B6-*mi/mi*, B6-*MI^{pr}/MI^{pr}*, B6-*mi^{ew}/mi^{ew}*, B6-*mi^{ce}/mi^{ce}*, and B6-*tg/tg* mice, but was normal in CMCs of B6-*MI^{wh}/MI^{wh}* mice.^{19–21,26–28} We attributed the decrease of skin mast cell number to the reduced level of KIT expression.

From the viewpoint of mast cell development, skin is an exceptional tissue because mast cells develop before birth only in the skin.^{26,29} To analyze the general mechanism for development of mast cells, studies using tissues other than skin may be necessary. We recently examined the number of mast cells in the peritoneal cavity of various MTF mutants.²⁹ In contrast to skin mast cells, peritoneal mast cells developed 6 weeks after birth

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The products of three genes have been known to influence the development of mast cells. Stem cell factor

even in B6-+/+ mice.²⁹ Mast cells never developed in the peritoneal cavity of B6-*mi/mi*, B6-*MI^{or}/MI^{or}*, B6-*mi^{ew}/mi^{ew}*, B6-*mi^{ce}/mi^{ce}*, B6-*MI^{wh}/MI^{wh}*, and B6-*tg/tg* mice.²⁹

We found a new mast cell adhesion molecule, spermatogenic immunoglobulin superfamily (SgIGSF).^{30,31} SgIGSF was expressed by CMCs derived from B6-+/+ mice, but not by CMCs from B6-*mi/mi*, B6-*MI^{or}/MI^{or}*, B6-*mi^{ew}/mi^{ew}*, B6-*mi^{ce}/mi^{ce}*, B6-*MI^{wh}/MI^{wh}*, or B6-*tg/tg* mice.³¹ To confirm the parallelism of SgIGSF expression and the number of peritoneal mast cells, we used B6-*mi^{vit}/mi^{vit}* mutant mice in the present experiment. All B6-*mi/mi*, B6-*MI^{or}/MI^{or}*, B6-*mi^{ew}/mi^{ew}*, B6-*mi^{ce}/mi^{ce}*, B6-*MI^{wh}/MI^{wh}*, and B6-*tg/tg* mice have a white coat color and small eyes, but B6-*mi^{vit}/mi^{vit}* mice have a black coat with white patches on the belly and thorax and eyes of normal size.^{24,32,33} We found that the magnitude of SgIGSF expression in CMCs derived from B6-*mi^{vit}/mi^{vit}* mice was half that of B6-+/+ mice and that the number of peritoneal mast cells in B6-*mi^{vit}/mi^{vit}* mice was one-sixth that of B6-+/+ mice.

Materials and Methods

Mice and Cells

The B6-*tg/tg* and *mi/mi* mice were described previously.²⁸ Female B6-*mi^{vit}/+* mice and male B6-*mi^{vit}/mi^{vit}* mice were mated, and the resulting B6-*mi^{vit}/+* or B6-*mi^{vit}/mi^{vit}* mice were selected by their coat color; B6-*mi^{vit}/+* mice had a black coat, whereas B6-*mi^{vit}/mi^{vit}* mice had a black coat with white patches in the belly and thorax.³³ (WB × B6)F₁ (WBB6F₁)-*W/W'* mice were purchased from the Japan SLC (Hamamatsu, Japan). CMCs were maintained in α -minimal essential medium (α -MEM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10% fetal calf serum (Nippon Bio-Supp Center, Tokyo, Japan) and 10% pokeweed mitogen-stimulated spleen cell conditioned medium as mentioned before.³⁴ Transfection of CMCs with a retrovirus vector containing SgIGSF cDNA was performed as described previously.³² The MST cells, kindly provided by Dr. J. D. Esko (University of California, San Diego, CA),³⁵ were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum. The NIH/3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories, Irvine, UK) supplemented with 10% fetal calf serum.

Staining and Counting of Mast Cells

Twelve weeks after birth, mice were killed by decapitation after ether anesthesia. Mast cell numbers in the peritoneal cavity, skin, glandular stomach, and mesentery were estimated as described previously.²⁹ In brief, Tyrode's buffer containing 0.1% gelatin (Sigma Chemical Co.) was injected into the peritoneal cavity, and the fluid containing the peritoneal cells was aspirated with a Pasteur pipette. After centrifugation, the pellet was resuspended with the Tyrode's buffer, and the peritoneal cells were attached to a microscope slide with a Cytospin 2 centrifuge (Shan-

don, Pittsburgh, PA). Pieces of dorsal skin and glandular stomach were removed and smoothed onto a piece of the filter paper to keep them flat. Mesentery was also smoothed onto a microscope slide. All specimens were fixed in Carnoy's solution. The cytospin preparation of peritoneal cells, the sections of skin and glandular stomach, and the stretch preparation of mesentery were stained with Alcian blue and nuclear fast red.

Northern Blot Analysis

Total RNAs (20 μ g) isolated with the lithium chloride-urea method³⁶ were used for Northern blot. The fragments of mMCP-4,³⁷ mMCP-5,³⁸ mMCP-6,³⁹ KIT,⁴⁰ tryptophan hydroxylase (TPH),¹⁴ SgIGSF,³¹ and β -actin⁴¹ cDNAs were labeled with [³²P]- α -dCTP (10 mCi/ml; DuPont/NEN Research Products, Boston, MA) by random oligonucleotide priming. After hybridization at 42°C, blots were washed to a final stringency of 0.2× standard saline citrate (1× standard saline citrate is 150 mmol/L NaCl and 15 mmol/L trisodium citrate, pH 7.4) and subjected to autoradiography.

Immunoblot Analysis

Peritoneal mast cells for immunoblot analysis were purified from cells in the peritoneal cavity with metrizamide as described previously.⁴² CMCs and peritoneal mast cells were lysed in a buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% Triton X-100, and 1 mmol/L phenylmethyl sulfonyl fluoride. The resulting lysates were separated on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to Immobilon (Millipore, Bedford, MA), and reacted with the anti-SgIGSF antibody made at Kanazawa University (by TW and SI).³⁰ After washing, the blots were incubated with an appropriate peroxidase-labeled secondary antibody, and then reacted with Renaissance reagents (NEN, Boston, MA) before exposure. After stripping, the blots were probed with the anti-actin antibody (Sigma Chemical Co.).

Attachment of CMCs to NIH/3T3 Fibroblasts

Co-culture of CMCs with NIH/3T3 cells was performed as described previously.³¹ Briefly, CMCs (1.0×10^5 cells per dish) were suspended in 2 ml of α -MEM containing 10% pokeweed mitogen-stimulated spleen cell conditioned medium and 10% fetal calf serum, and then added to a confluent culture of NIH/3T3 cells in 35-mm culture dishes. After 3 hours of co-culture, the dishes were washed with warmed (37°C) α -MEM to remove nonadherent CMCs. NIH/3T3 cells and adherent CMCs were harvested by trypsin treatment. These cells were attached to microscope slides using the Cytospin 2 centrifuge, fixed with Carnoy's solution, and stained with Alcian blue and nuclear fast red. The proportion of Alcian blue-positive mast cells to Alcian blue-negative NIH/3T3 cells was determined.

Intraperitoneal Injection of CMCs

CMCs (1.0×10^6) derived from B6-+/+, B6-*tg/tg*, or B6-*mi^{vit}/mi^{vit}* mice were suspended in 0.5 ml of α -MEM, and were injected into the peritoneal cavity of WBB6F₁-W/W^v mice. In a separate experiment, CMCs derived from B6-*tg/tg* or B6-*mi^{vit}/mi^{vit}* mice and those overexpressing SgIGSF cDNA or empty vector alone were injected into the peritoneal cavity of B6-*tg/tg* mice. Five weeks after injection, peritoneal cells were harvested, and proportions of mast cells in 1000 peritoneal cells were determined as described above.

Subcellular Localization

The pEGFP3B expression vector containing EGFP cDNA was kindly provided by Dr. N. Yabuta (Osaka University, Osaka, Japan).⁴³ The fragment containing the coding region of normal MITF (+-MITF), MITF encoded by the *mi^{vit}* or *mi* mutant allele (*mi^{vit}*-MITF and *mi*-MITF, respectively) was amplified by Pyrobest TaqDNA polymerase (Takara, Kyoto, Japan), and was cloned into the blunted AclI site of pEGFP3B. The sequence of constructs was verified with ABI 3100 sequencer (Applied BioSystems, Foster City, CA) in both directions. The expression plasmid was transfected into NIH/3T3 cells, and the subcellular localization of MITF protein fused with EGFP was detected with a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Jena, Germany).

Electrophoretic Gel Mobility Shift Assay

The production of the fusion protein containing glutathione S-transferase (GST) and MITF was described previously.⁴⁴ To examine the DNA binding ability of MITF, an oligonucleotide that is a part of SgIGSF promoter was used as a probe.³¹ The sequence of the oligonucleotide is 5'-GCTTTAATGTGTAAGTCAATTTGATGGGTTGGCCGA (the sequence recognized by +-MITF was underlined). The oligonucleotide was labeled with α -[³²P]-dCTP by filling 5'-overhangs and used as probes of electrophoretic gel mobility shift assay. DNA-binding assays were performed in a 20- μ l reaction mixture containing 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L ethylenediaminetetraacetic acid, 75 mmol/L KCl, 1 mmol/L dithiothreitol, 4% Ficoll type 400, 50 ng of poly (dI-dC), 25 ng of labeled DNA probe, and 3.5 μ g of GST-MITF fusion protein. After the incubation at 37°C for 15 minutes, the reaction mixture was subjected to electrophoresis at 14 volt/cm at 4°C on a 5% polyacrylamide gel in 0.25 $\times$ Tris-borate-ethylenediaminetetraacetic acid buffer (1 $\times$ Tris-borate-ethylenediaminetetraacetic acid is 90 mmol/L Tris-HCl (Sigma), 64.6 mmol/L boric acid (Sigma), and 2.5 mmol/L ethylenediaminetetraacetic acid (Sigma), pH 8.3). The polyacrylamide gels were dried on Whatman 3MM chromatography paper and subjected to autoradiography.

Dissociation Assay

Dissociation assay was performed as described previously.¹⁹ Reaction mixtures for the DNA-+-MITF and DNA-

Figure 1. Schematic diagram of MITF structure. The basic, helix, and Zip domains are shown. Mutated points in MITF encoded by various mutant alleles are also indicated. a.a., Amino acid; I, isoleucine; N, asparagine; R, arginine; K, lysine; Δ R, deletion of arginine; D, aspartic acid; stop, stop codon.

mi^{vit}-MITF complexes were prepared as described above. A 100-fold molar excess of nonlabeled oligonucleotide containing the CATTG motif was added, and the reaction mixtures were electrophoresed after incubation times of 1, 2, and 4 minutes. The intensity of the band for DNA-MITF complex was quantified with NIHimage, and the values were plotted against the incubation period.

Luciferase Assay

The reporter plasmid containing the promoter region of SgIGSF gene (nucleotides -1501 to +19, +1 is a transcription initiation site) was described previously.³¹ The effector plasmids were constructed by introducing the fragment of coding region of +-, *mi*-, and *mi^{vit}*-MITF cDNAs into the pEF-BOS expression vector kindly provided by Dr. S. Nagata (Osaka University Medical School, Osaka, Japan).⁴⁵ Five μ g of a reporter, 0.1 μ g of an effector plasmid, and 1 μ g of an expression vector containing β -galactosidase gene were co-transfected into MST cells by electroporation. The expression vector containing the β -galactosidase gene was used as an internal control. The cells were harvested 48 hours after the transfection, and the soluble extracts were assayed for luciferase activity and for β -galactosidase activity as described previously.¹⁰ The normalized value by the β -galactosidase activity was expressed as the relative luciferase activity.

Results

We previously investigated various MITF mutant mice. The B6-*tg/tg* mice do not express any MITFs because of an insertion mutation in the promoter region (Figure 1).⁷ The *mi*-MITF, *mi^{or}*-MITF, and *mi^{wh}*-MITF are mutated at a single amino acid in the basic domain (Figure 1).²⁴ The *mi^{ew}*-MITF deletes a large portion of the basic domain, and *mi^{ce}*-MITF deletes that of the Zip domain (Figure 1).²⁴ The coat color of B6-*mi/mi*, B6-*mi^{or}/mi^{or}*, B6-*mi^{wh}/mi^{wh}*, B6-*mi^{ew}/mi^{ew}*, B6-*mi^{ce}/mi^{ce}*, and B6-*tg/tg* mice is white because of a lack of melanocytes, and the microphthalmia are observed in all of the above-mentioned six mutant mice (Figure 2).^{24,32} The *mi^{vit}*-MITF is mutated at a

Figure 2. Appearance of B6-+/+, B6-*tg/tg*, and B6-*mi^{vit}/mi^{vit}* mice. B6-*tg/tg* mouse has the white coat and microphthalmia, whereas B6-*mi^{vit}/mi^{vit}* mouse has the black coat with white patches in thorax and abdomen and the eyes of normal size.

single amino acid in the helix domain (Figure 1).²⁴ B6-*mi^{vit}/mi^{vit}* mice have a black coat with white patches on the belly and thorax and eyes of normal size (Figure 2).

We counted the number of mast cells in tissues of B6-*mi^{vit}/mi^{vit}* mice. Because mast cells in tissues other than skin develop 6 weeks after birth,²⁹ we examined the mast cell number using B6-*mi^{vit}/mi^{vit}* mice of 12 weeks of age. The number of mast cells in the skin, glandular stomach, and mesentery of B6-*mi^{vit}/mi^{vit}* mice was comparable to that of B6-+/+ mice, but the number of mast cells in the peritoneal cavity decreased to one-sixth that of B6-+/+ mice (Table 1). The number of skin mast cells in B6-*tg/tg* mice decreased to one-third that of B6-+/+ mice, and mast cells were depleted in the glandular stomach, mesentery, and peritoneal cavity of B6-*tg/tg* mice (Table 1).

We compared the expression levels of various genes in CMCs derived from B6-*mi^{vit}/mi^{vit}* mice with those of B6-*tg/tg*, B6-*mi/mi*, and B6-+/+ mice. As described previously,²⁸ the expression level of mMCP-4, mMCP-5, mMCP-6, and SgIGSF genes decreased remarkably, and that of KIT and TPH genes decreased moderately in B6-*tg/tg* CMCs (Figure 3). In B6-*mi/mi* CMCs, the expression level of all examined genes decreased remarkably (Figure 3). The expression levels of mMCP-4, mMCP-5, mMCP-6, KIT, and TPH genes were comparable between B6-*mi^{vit}/mi^{vit}* CMCs and B6-+/+ CMCs. However, the expression level of SgIGSF gene of B6-*mi^{vit}/mi^{vit}* CMCs

Figure 3. Expression of various genes in CMCs derived from B6-+/+, B6-*tg/tg*, B6-*mi/mi*, and B6-*mi^{vit}/mi^{vit}* mice. The blot was hybridized with ³²P-labeled cDNA probe of SgIGSF, mMCP-4, mMCP-5, mMCP-6, KIT, TPH, and β -actin. Three independent experiments were performed, and comparable results were obtained. A representative experiment is shown.

was intermediate between the level of B6-+/+ CMCs and the levels of B6-*tg/tg* and B6-*mi/mi* CMCs (Figure 3).

The expression of SgIGSF was also examined at the protein level by immunoblot. The signal of SgIGSF was hardly detected in B6-*tg/tg* and B6-*mi/mi* CMCs. On the other hand, the SgIGSF signal was apparently detectable in B6-*mi^{vit}/mi^{vit}* CMCs but was significantly reduced when compared to that of B6-+/+ CMCs (Figure 4).

Table 1. Number of Mast Cells in Various Tissues of B6-+/+, -*mi^{vit}/mi^{vit}*, or -*tg/tg* Mice

Genotype of mice	Number of mast cells*			
	Skin [†]	Glandular stomach [‡]	Mesentery [§]	Peritoneal cavity [¶]
B6-+/+	159 ± 15	135 ± 9	6.9 ± 0.8	33.7 ± 1.5
B6- <i>mi^{vit}/mi^{vit}</i>	160 ± 14	123 ± 10	5.2 ± 0.5	5.6 ± 1.4
B6- <i>tg/tg</i>	62 ± 12	ND ^{**}	ND ^{**}	ND ^{**}

*Mean ± SE of seven mice.

[†]Number of mast cells per cm of skin section.

[‡]Number of mast cells per cm of stomach section.

[§]Number of mast cells per mm² of stretched mesentery.

[¶]Number of Alcian blue-positive cells per 10³ nucleated peritoneal cells.

^{||}*P* < 0.01 when compared with the value of +/+ mice by *t*-test.

^{**}Not detectable.

Figure 4. Immunoblot of SgIGSF in CMCs derived from B6-+/+, B6-*tg/tg*, B6-*mi/mi*, and B6-*mi^{vit}/mi^{vit}* mice. Whole cell extracts of CMCs were immunoblotted with anti-SgIGSF antibody. Three independent experiments were performed and a representative result is shown.

SgIGSF is important for the attachment of CMCs to NIH/3T3 fibroblasts.³¹ We cultured B6-*mi^{vit}/mi^{vit}* CMCs on the monolayer of NIH/3T3 cells. After 3 hours of the co-culture, the number of adhering B6-*mi^{vit}/mi^{vit}* CMCs per NIH/3T3 cell was counted. The number of adhering B6-*mi^{vit}/mi^{vit}* CMCs was intermediate between the number of B6-+/+ CMCs and the numbers of B6-*tg/tg* and B6-*mi/mi* CMCs (Table 2). The overexpression of SgIGSF cDNA in B6-*mi^{vit}/mi^{vit}* CMCs increased the number of adhering CMCs to the level comparable to that of B6-+/+ CMCs (Table 3).

The expression of SgIGSF was examined in mast cells obtained from the peritoneal cavity. As observed in CMCs, the SgIGSF signal was apparently detectable in the peritoneal mast cells derived from B6-*mi^{vit}/mi^{vit}* mice, but its signal was reduced as compared to that of the peritoneal mast cells derived from B6-+/+ mice (Figure 5).

We previously found that intraperitoneally injected B6-+/+ but not B6-*tg/tg* CMCs survived in the peritoneal cavity of mast cell-deficient WBB6F₁-*W/W^v* mice.²⁹ To examine the correlation between SgIGSF expression and survival of CMCs in the peritoneal cavity, we injected B6-+/+, B6-*tg/tg*, and B6-*mi^{vit}/mi^{vit}* CMCs to the peritoneal cavity of WBB6F₁-*W/W^v* mice. Five weeks after in-

Table 2. Attachment of CMCs to NIH/3T3 Fibroblasts

Genotype of CMCs	No. of adhering CMCs per NIH/3T3 cell*
B6-+/+	0.149 ± 0.002 [†]
B6- <i>tg/tg</i>	0.068 ± 0.002 [‡]
B6- <i>mi/mi</i>	0.062 ± 0.002 [‡]
B6- <i>mi^{vit}/mi^{vit}</i>	0.094 ± 0.003 ^{†‡}

*Mean ± SE of three dishes.

[†]*P* < 0.01 by *t*-test when compared with the values of B6-*tg/tg* CMCs.

[‡]*P* < 0.01 by *t*-test when compared with the values of B6-+/+ CMCs.

Table 3. Effect of SgIGSF on the Attachment of B6-*mi^{vit}/mi^{vit}* CMCs to NIH/3T3 Fibroblasts

Genotype of CMCs	No. of adhering CMCs per NIH/3T3 cell*
B6-+/+	0.170 ± 0.006 [†]
B6- <i>tg/tg</i>	0.082 ± 0.003 [‡]
B6- <i>mi^{vit}/mi^{vit}</i> overexpressing empty vector	0.115 ± 0.004 ^{†‡}
B6- <i>mi^{vit}/mi^{vit}</i> overexpressing SgIGSF cDNA	0.190 ± 0.004 [†]

*Mean ± SE of three dishes.

[†]*P* < 0.01 by *t*-test when compared with the values of B6-*tg/tg* CMCs.

[‡]*P* < 0.01 by *t*-test when compared with the values of B6-+/+ CMCs.

jection, the number of surviving mast cells was counted. As reported previously,²⁹ the number of surviving B6-*tg/tg* CMCs was significantly lower than that of surviving B6-+/+ CMCs (Table 4). However, no difference was observed between the number of surviving B6-+/+ CMCs and that of surviving B6-*mi^{vit}/mi^{vit}* CMCs (Table 4).

B6-+/+ CMCs but not B6-*tg/tg* CMCs survived also in the peritoneal cavity of B6-*tg/tg* mice.²⁹ Then, we injected B6-+/+ CMCs, B6-*tg/tg* CMCs, or B6-*tg/tg* CMCs overexpressing SgIGSF cDNA to the peritoneal cavity of B6-*tg/tg* mice. As previously reported, the number of surviving B6-*tg/tg* CMCs was significantly lower than the number of surviving B6-+/+ CMCs. However, the number of surviving B6-*tg/tg* CMCs substantially increased when SgIGSF cDNA was overexpressed but not when the empty vector alone was overexpressed (Table 5). B6-*mi^{vit}/mi^{vit}* CMCs or B6-*mi^{vit}/mi^{vit}* CMCs overexpressing SgIGSF cDNA were also injected into the peritoneal cavity of B6-*tg/tg* mice. The surviving mast cell number was comparable among the cases when B6-+/+ CMCs, B6-

Figure 5. Immunoblot of SgIGSF in peritoneal mast cells derived from B6-+/+ and B6-*mi^{vit}/mi^{vit}* mice. Whole cell extracts of peritoneal mast cells were immunoblotted with anti-SgIGSF antibody. Two independent experiments were performed, and a representative result is shown.

Table 4. Number of Mast Cells in the Peritoneal Cavity of WBB6F₁-W/W^v Mice Injected with B6-+/+, B6-*mi^{vit}/mi^{vit}*, and B6-*tg/tg* CMCs 5 Weeks after the Injection

Origin of injected CMCs	No. of mast cells*†
B6-+/+	25.5 ± 3.2
B6- <i>tg/tg</i>	5.5 ± 0.6‡
B6- <i>mi^{vit}/mi^{vit}</i>	21.0 ± 4.4

*Mean ± SE of five to seven mice.

†Number of Alcian blue-positive cells per 10³ nucleated peritoneal cells.

‡*P* < 0.01 by *t*-test when compared with the value in the case that B6-+/+ CMCs were injected.

mi^{vit}/mi^{vit} CMCs, or B6-*mi^{vit}/mi^{vit}* CMCs overexpressing SgIGSF cDNA were injected (Table 5).

The molecular nature of *mi^{vit}*-MITF was examined in the following three aspects: subcellular localization, DNA binding, and transactivation potential for the promoter region of SgIGSF gene. First, we examined the subcellular localization of *mi^{vit}*-MITF by using the fusion protein with EGFP. When the expression vector containing EGFP cDNA alone was transfected to NIH/3T3 cells, the EGFP protein was detected in both nucleus and cytoplasm (Figure 6). We reported that the +MITF localized to the nucleus and that *mi*-MITF did not.⁴⁶ Consistent with this, the EGFP fused with +MITF was localized to the nucleus, and EGFP fused with *mi*-MITF was detected in both nucleus and cytoplasm (Figure 6). The EGFP fused with *mi^{vit}*-MITF localized to the nucleus as in the case of +MITF (Figure 6).

Next, the DNA binding ability of *mi^{vit}*-MITF was examined by electrophoretic gel mobility shift assay. The part of the SgIGSF promoter containing the MITF-binding motif, CATTG,³¹ was used as the probe. As reported previously,³¹ the specific binding of +MITF to the CATTG motif was detected, but that of *mi*-MITF was not (Figure 7A). The specific binding of *mi^{vit}*-MITF was also observed. However, the amount of DNA-*mi^{vit}*-MITF complex was smaller than that of DNA-+MITF complex (Figure 7A). When the dissociation assay was performed, the dissociation rate of DNA-*mi^{vit}*-MITF complex was higher than that of DNA-+MITF complex (Figure 7, B and C). In

Table 5. Number of Mast Cells in the Peritoneal Cavity of B6-*tg/tg* Mice Injected with B6-+/+, B6-*tg/tg*, B6-*mi^{vit}/mi^{vit}* CMCs and B6-*tg/tg* or B6-*mi^{vit}/mi^{vit}* CMCs Overexpressing SgIGSF

Origin of injected CMCs	No. of mast cells*†
B6-+/+	28.0 ± 5.2‡
B6- <i>tg/tg</i>	1.9 ± 0.6
B6- <i>tg/tg</i> overexpressing empty vector	1.6 ± 0.4
B6- <i>tg/tg</i> overexpressing SgIGSF cDNA	10.7 ± 3.6‡
B6- <i>mi^{vit}/mi^{vit}</i>	24.9 ± 2.5‡
B6- <i>mi^{vit}/mi^{vit}</i> overexpressing SgIGSF cDNA	27.7 ± 2.8‡

*Mean ± SE of five to seven mice.

†Number of Alcian blue-positive cells per 10³ nucleated peritoneal cells.

‡*P* < 0.01 by *t*-test when compared with the value in the case that B6-*tg/tg* CMCs were injected.

Figure 6. Subcellular localization of *mi^{vit}*-MITF. MITF fused with GFP was expressed in NIH/3T3 cells, and its subcellular localization was examined. A NIH/3T3 cell transfected with GFP expression vector is shown as vec, and NIH/3T3 cells expressing +MITF, *mi*-MITF, or *mi^{vit}*-MITF fused with GFP are shown as wt, *mi*, and *vit*, respectively.

other words, the affinity of *mi^{vit}*-MITF to DNA was lower than that of +MITF.

We examined the effect of *mi^{vit}*-MITF on the transactivation of SgIGSF gene using the co-transfection assay. We have reported that the +MITF but not *mi*-MITF transactivates the SgIGSF promoter by binding the CATTG motif located between nucleotides -1490 and -1485.³¹ The reporter plasmid containing this CATTG motif was co-transfected into MST mastocytoma cells with an empty pEF-BOS plasmid or with the vectors expressing +MITF, *mi*-MITF, or *mi^{vit}*-MITF. Co-transfection with the +MITF cDNA increased the luciferase activity 3.5-fold as strongly as co-transfection with the empty vector (Figure 8). The luciferase activity obtained by co-transfection with the *mi^{vit}*-MITF cDNA was intermediate between that of +MITF cDNA and that of empty vector (Figure 8). As reported previously,³¹ the luciferase activity by co-transfection with the *mi*-MITF cDNA was comparable to that of empty vector.

Discussion

B6-*tg/tg* mice do not express MITF because of the insertion mutation at the promoter region, and therefore they are useful as a standard of MITF mutants.^{20,21,28} B6-*tg/tg* mice have white hair and small eyes.²⁴ The number of mast cells decreased to one-third in the skin of B6-*tg/tg* mice, and no mast cells were detectable in the stomach, mesentery, and peritoneal cavity of B6-*tg/tg* mice.²⁹ B6-*mi^{vit}/mi^{vit}* mice have black hair at least at young age, and do not show apparent microphthalmia.^{24,33} In contrast to B6-*tg/tg* mice, B6-*mi^{vit}/mi^{vit}* mice had mast cells in the peritoneal cavity, but their number decreased to one-sixth. When phenotypes are compared between B6-*tg/tg* and B6-*mi^{vit}/mi^{vit}* mice, those of B6-*tg/tg* mice were more severe than those of B6-*mi^{vit}/mi^{vit}* mice, indicating *mi^{vit}*-MITF possessed an appreciable transactivation potential despite the single amino acid alteration at the helix domain.

Figure 7. Reduced DNA binding ability of *mi^{vlt}*-MITF. **A:** Electrophoretic gel mobility shift assay using GST fusion protein of +MITF, *mi^{vlt}*-MITF, or *mi*-MITF. An approximately equal amount of GST-+-MITF, GST-*mi^{vlt}*-MITF, or GST-*mi*-MITF was added in each lane, and was verified by immunoblot with anti-GST antibody. **B:** Dissociation assay. The DNA-+-MITF and DNA-*mi^{vlt}*-MITF complexes were formed, and the cold competitor was added. The reaction mixtures were electrophoresed after incubation times of 1, 2, and 4 minutes. **C:** Plots of the intensity of DNA-+-MITF and DNA-*mi^{vlt}*-MITF complexes against the incubation period.

A deletion of an amino acid (*mi* allele) or alteration of an amino acid (*Mr^{pr}* allele) at the basic domain results in more severe phenotypes than the null mutation caused by the *tg* mutant allele.^{19,28} Another alteration of an amino acid at the basic domain (*Mr^{wh}* allele) resulted in a rather moderate phenotype in the homozygote.^{19,47} However, the B6-*Mr^{wh}*/+ heterozygote shows an apparently domi-

Figure 8. Transactivation by *mi^{vlt}*-MITF. The reporter plasmid contained the SglGSF promoter starting from nucleotide -1501. The effector plasmid contained +MITF, *mi^{vlt}*-MITF, or *mi*-MITF cDNA. The reporter plasmid and the effector plasmid were co-transfected to MST cells, and the luciferase activity was measured. The values represent the mean $\pm$ SE of three experiments. In some cases, the SE 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 +MITF.

nant-negative phenotype, ie, the remarkable dilution of coat color.^{24,32} The heterozygous mouse of B6-*mi*/+, B6-*Mr^{pr}*/+, B6-*tg*/+, or B6-*mi^{vlt}*/+ genotype shows very weak or null phenotype in B6 genetic background.^{7,24,32} Taken together, in both homozygous and heterozygous conditions, the *mi^{vlt}* mutant allele shows the weakest effect in all of the above-mentioned MITF mutant alleles.

The DNA binding potential of *mi^{vlt}*-MITF was apparently detectable but its affinity to DNA was weaker than that of +MITF. This was not consistent with the finding of Hemesath and colleagues²⁵ that *mi^{vlt}*-MITF normally bind to the adenovirus major late promoter having the CACGTG motif as a core sequence. We produced *mi^{vlt}*-MITF as a GST fusion protein in *Escherichia coli*, whereas Hemesath and colleagues²⁵ produced *mi^{vlt}*-MITF using reticulocyte lysate. We suppose that the purity of *mi^{vlt}*-MITF used in our experiment was higher than that of Hemesath and colleagues,²⁵ because we purified the GST-*mi^{vlt}*-MITF fusion protein by affinity chromatography on immobilized glutathione. The detection of the affinity to DNA may be more sensitive when GST-*mi^{vlt}*-MITF fusion protein was used than when *mi^{vlt}*-MITF obtained from reticulocyte lysate was used. There is another possibility that the difference between the result of Hemesath and colleagues²⁵ and our result may be attributable to the difference of probes.

Steingrimsson and colleagues⁴⁸ reported another mutant allele at the helix domain (*Mr^p*). Although *Mr^p*-MITF also has a single amino acid alteration, homozygous *Mr^p*/*Mr^p* mice have white hair,⁴⁸ indicating that their phenotype was more severe than that of B6-*mi^{vlt}*/*mi^{vlt}* mice. Although both *Mr^p*-MITF and *mi^{vlt}*-MITF have a single amino acid alteration in helix domain, the functions of *Mr^p*-MITF and *mi^{vlt}*-MITF were different; the DNA binding ability was abolished in *Mr^p*-MITF⁴⁸ but detectable in *mi^{vlt}*-MITF. The amino acid mutated in *Mr^p*-MITF is supposed to locate at the position close to the protein-DNA interface.⁴⁸ In contrast, the amino acid mutated in *mi^{vlt}*-MITF is supposed to lie on the outside face of the helix domain, which is not so close to the protein-DNA interface.²⁴ The difference of the affinity to DNA between *Mr^p*-MITF and *mi^{vlt}*-MITF may be explained by the different location of the mutated amino acid.

Young B6-*mi^{vlt}*/*mi^{vlt}* mice have black hair in the pigmented area, but gradual depigmentation occurs.³³ This is because of the progressive loss of melanocytes.³³ In con-

trast to the progressive decrease of melanocytes, mast cell number in the skin of B6-*mi^{vit}/mi^{vit}* mice was comparable to that of B6-+/+ mice either at 2 weeks of age (unpublished data) or at 12 weeks of age. The effect of *mi^{vit}*-MITF on mast cells was different from that of melanocytes.

The expression levels of mMCP-4, mMCP-5, mMCP-6, KIT, and TPH genes were comparable between B6-*mi^{vit}/mi^{vit}* and B6-+/+ CMCs, but the expression level of SgIGSF gene was lower in B6-*mi^{vit}/mi^{vit}* CMCs than in B6-+/+ CMCs. The requirement of MITF for the expression of SgIGSF gene appeared to be higher than the requirement of MITF for the expression of mMCP-4, mMCP-5, mMCP-6, KIT, and TPH genes.

The expression level of SgIGSF in B6-*mi^{vit}/mi^{vit}* CMCs was intermediate between that of B6-+/+ CMCs and that of B6-*tg/tg* CMCs. The adherence potential of B6-*mi^{vit}/mi^{vit}* CMCs to NIH/3T3 fibroblasts was also intermediate between that of B6-+/+ CMCs and that of B6-*tg/tg* CMCs. The adherence of CMCs to NIH/3T3 fibroblasts appeared to be mediated by SgIGSF, because the overexpression of SgIGSF in B6-*mi^{vit}/mi^{vit}* CMCs increased the adherence potential to the level comparable to that of B6-+/+ CMCs.

Although B6-*tg/tg* mice lacked peritoneal mast cells, B6-*mi^{vit}/mi^{vit}* mice possessed the significantly reduced but apparently detectable number of peritoneal mast cells. The SgIGSF expression of detectable level in CMCs and in peritoneal mast cells of B6-*mi^{vit}/mi^{vit}* mice appeared to be correlated with the presence of mast cells in the peritoneal cavity of B6-*mi^{vit}/mi^{vit}* mice.

The number of mast cells in mesentery did not decrease in B6-*mi^{vit}/mi^{vit}* mice. B6-*Mi^{wh}/Mi^{wh}* mice, which abolished SgIGSF expression in CMCs, also had a normal number of mast cells in the mesentery (unpublished data). The expression of SgIGSF did not appear to be correlated with development of mast cells in the mesentery. Mendonca and colleagues⁴⁹ reported that the development of mast cells in the peritoneal cavity and that of the mesentery were independent of each other. The expression of SgIGSF may be essential for the former case but not for the latter case.

In the peritoneal cavity of B6-*tg/tg* mice, the number of surviving B6-*tg/tg* CMCs was higher when SgIGSF was overexpressed than when the empty vector was overexpressed. This indicated that SgIGSF played an important role for the survival of CMCs in the peritoneal cavity. However, the ectopic expression of SgIGSF did not completely restore the poor survival of B6-*tg/tg* CMCs. SgIGSF might require some molecules for supporting the survival of CMCs. B6-*tg/tg* CMCs expressed an appreciable amount but still significantly reduced level of KIT.²⁸ Normal expression of KIT might be prerequisite for SgIGSF to support the survival of CMCs.

Although peritoneal mast cells of B6-*mi^{vit}/mi^{vit}* mice reduced in number, the injected B6-*mi^{vit}/mi^{vit}* CMCs survived normally in the peritoneal cavity of WBB6F₁-*W/W^c* or B6-*tg/tg* mice. This suggested the following two possibilities. First, the amount of SgIGSF required for survival of CMCs in peritoneal cavity was different from that required for development of mast cells in peritoneal cavity. The SgIGSF expression level of B6-*mi^{vit}/mi^{vit}* CMCs may

be sufficient for the survival of CMCs. Second, some molecule(s) other than SgIGSF was normally expressed in B6-+/+ and B6-*mi^{vit}/mi^{vit}* CMCs but not in B6-*tg/tg* CMCs. This molecule(s) might play an important role for the survival in peritoneal cavity. As discussed above, KIT was one of the candidates for such molecules.

Growth factor receptors and adhesion molecules are important for development of cells derived from hematopoietic stem cells.⁵⁰ Because the expression of both growth factor receptors and that of adhesion molecules are regulated by transcription factors, the particular set of transcription factors are considered to regulate the number of certain hematopoietic cells. Signals through KIT receptors are essential for development of mast cells in tissues.^{1,2,4} The expression of SgIGSF is also necessary for development of mast cells in tissues other than skin. The expression of both KIT and SgIGSF is regulated by MITF.^{11,31} Thus, MITF appears to be one of transcription factors that regulate the number of mast cells in tissues. MITF mutant mice are a useful model for studying regulation of mast cell number by transcription factors.

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