MITF Is Necessary for Generation of Prostaglandin D$_2$ in Mouse Mast Cells*

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Mast cells generate eicosanoids that are linked to asthma and other inflammatory diseases. A basic-helix-loop-helix leucine zipper transcription factor termed MITF is essential for the development of mast cells. Although other substances also linked to inflammatory reactions (such as various proteases and serotonin) require MITF for their expression, the role of MITF in eicosanoid generation has not been studied. We examined eicosanoid generation in bone marrow-derived mast cells (BMMCs) of $tg/tg$ mice that lack MITF. Most eicosanoids generated by BMMCs are either prostaglandin (PG) D$_2$ or leukotriene C$_4$. The former is synthesized via the cyclooxygenase pathway, whereas the latter is synthesized via the 5-lipoxygenase pathway. In response to stimulation with IgE and antigens, BMMCs of $tg/tg$ mice synthesized leukotriene C$_4$ normally. However, neither immediate nor delayed PGD$_2$ production was detected in these BMMCs. This indicates that MITF is a transcription factor that specifically activates the cyclooxygenase pathway, but not the 5-lipoxygenase pathway. Significant decreases in expression of hematopoietic PGD$_2$ synthase (hPGDS, a terminal synthase for cyclooxygenase pathway, but not the 5-lipoxygenase pathway. The immediate generation of PGD$_2$ from PGHS-1 and -2 and is unstable, whereas the delayed synthesis of PGD$_2$ is mediated by constitutively expressed PGHS. PGH$_2$ is generated in BMMCs by both PGHS-1 and -2 and is unstable, quickly being metabolized to PGD$_2$ by hematopoietic PGD$_2$ synthase (hPGDS) (21). Recently, Stevens and coworkers (22) demonstrated that Ras guanine nucleotide-releasing protein (RasGRP) 4 regulates expression of the hPGDS gene and that mast cell lines with low RasGRP4 expression levels show defective generation of PGD$_2$ but normal generation of LTC$_4$. RasGRP4 appeared to be involved in the cyclooxygenase pathway, but not in the 5-lipoxygenase pathway. In our study we examined eicosanoid generation in BMMCs of $tg/tg$ mice and found that PGD$_2$, but not LTC$_4$, synthesis requires MITF. MITF appears to be essential for the cyclooxygenase pathway and not in the 5-lipoxygenase pathway.

**Experimental Procedures**

**Mice and Cells**—The original stock of $tg/tg$ mice, in which the mouse vasopressin-<i>Escherichia coli</i> β-galactosidase transgene was integrated at the promoter region of the MITF gene, was provided by Dr. H. Arnheiter (National Institutes of Health, Bethesda, MD) (5). The $tg/tg$ mice were maintained by consecutive backcrosses to our own C57BL/6 (B6) and WB inbred colonies for more than 15 generations. Female B6-$tg/tg$ and male WB-$tg/tg$ mice were crossed, and the resulting (WB × B6) F$_1$ (WBB6F$_1$)-$tg/tg$ mice were selected by their white coat color. WBB6F$_1$-$tg/tg$ mice were purchased from Japan SLC (Hamamatsu, Japan). WBB6F$_1$-$tg/tg$ and WBB6F$_1$-+/+ mice were termed $+/+$ and $tg/tg$ mice, respectively. BMMCs were established from 4–6-week-old $+/+$ or $tg/tg$ mice by cultivating bone marrow cells with α-minimal essential medium (ICN Biomedicals, Costa Mesa, CA) containing 10 ng/ml recombinant mouse (rm) interleukin (IL-3) (R&D, Minneapolis, MN) for ~4–6 weeks. In some experiments, BMMCs established in the medium containing IL-3 were stimulated with α-minimal essential medium containing...

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1 The abbreviations used are: BMMC, bone marrow-derived mast cell; PGD$_2$, prostaglandin D$_2$; PGHS, PG endoperoxide H synthase; hPGD, hematopoietic PGD synthase; LTC$_4$, leukotriene C$_4$; KITL, Kit ligand; IL, interleukin; RasGRP, Ras guanine nucleotide-releasing protein; rm, recombinant mouse; DNP, dimethylnitrosamine; HSA, human serum albumin; GST, glutathione S-transferase.
taining rmIL-10 (10 ng/ml; R&D), rmIL-18 (5 ng/ml; R&D) and rmKitL (50 ng/ml; R&D) for 2 h.

Stimulation of BMMCs—BMMCs were suspended at a concentration of 1 × 10⁷ cells/ml in medium containing cytokines for eosinophil generation or in Tyrode’s buffer containing 1.8 mM Ca²⁺, 0.2 mM Mg²⁺, 0.4% (w/v) bovine serum albumin (type V; Sigma), and 10 mM Hepes (pH 7.2) for β-hexosaminidase release. The suspended cells were sensitized with 1 μg/ml anti-dinitrophenyl (DNP) IgE (Sigma) for 2 h, washed, and then elicited with various concentrations of DNP-conjugated human serum albumin (HSA; Sigma).

Measurement of β-Hexosaminidase Release and Generation of Eicosanoids—The assay for β-hexosaminidase release was performed 30 min after elicitation with DNP-HSA. BMMCs were precipitated, and β-hexosaminidase activity in the supernatant and in the cell pellets (after lysis by freeze-thawing) was quantitated by spectrophotometric analysis of the hydrolysis of p-nitrophenyl-β-D-acetamido-β-D-galactoside (Sigma). The percent release of β-hexosaminidase was calculated by the formula [S/(S + H₉₂₆₂)] where S and H are the β-hexosaminidase contents of equal portions of supernatant and cell pellet, respectively. PGD₂ and LTC₄ levels were quantified in the supernatant of elicited BMMCs using enzyme immunoassay kits according to the manufacturer’s instructions (Cytochrome Chemical Company, Ann Arbor, MI). In some experiments, the amount of PGD₂ generated was measured in BMMCs preincubated with 1 μM indomethacin, washed, and then elicited with DNP-HSA.

Transcript Analysis of Stimulated BMMCs of +/- or tg/tg Mice—The expression profiles of genes in +/- or tg/tg BMMCs stimulated with anti-DNP IgE alone or stimulated with anti-DNP IgE and then elicited with DNP-HSA for 30 min were examined with a CodeLink UniSet Mouse 20K (Amersham Biosciences) using 2 μg of total RNA extracted with an RNeasy kit (Qiagen, Valencia, CA). Experimental procedures, including the synthesis of double-stranded cDNA and biotin-labeled cRNA target, and the analysis of results were performed by Kurabo Co. Ltd. (Osaka).

Quantification of mRNA Levels by Real-time RT-PCR—RNA was extracted from BMMCs using a RNeasy kit (Qiagen) with DNase I treatment. BMMCs cultured under the following four conditions were used: unstimulated, sensitized with anti-DNP IgE alone, sensitized with anti-DNP IgE and elicited with DNP-HSA for 30 min, or sensitized with anti-DNP IgE and elicited with DNP-HSA for 120 min. The mRNA levels for RasGRP4, PGHS-1, PGHS-2, hPGDS, and glycoldehyde-3-phosphate dehydrogenase genes were verified using a TaqMan Universal PCR Master Mix and Assays-on-Demand primers from Applied Biosystems (Foster City, CA). The probes and primers used for cell cycle regulators were Assays-on-Demand gene products. The mRNA levels for each gene were normalized to that of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Immunoblot Analysis—BMMCs were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The resulting lysates were separated on 8.3%, 5 mM MgCl₂, 0.1 mM EDTA, and 40% glycerol, and stored until use.

Frozen nuclei (100 μg) were added to 100 μl of buffer containing 20 mM Hepes (pH 8.3), 5 mM MgCl₂, 0.1 mM EDTA, and 40% glycerol, and stored until use. Frozen nuclei (100 μl) were added to 100 μl of buffer containing 20 mM Hepes-KOH (pH 8.0), 25% glycerol, 10 mM MgCl₂, 0.2 mM KCl, 1.2 mM ATP, 0.6 mM CTP, and 0.6 mM GTP. After the addition of 40 units/ml RNase inhibitor and 100 μCi of [α-³²P]UTP, the mixture was incubated at room temperature for 30 min. The labeled RNA was passed through a QiAshredder column (Qiagen) and purified with a Qiagen RNeasy kit according to the manufacturer's protocol. The labeled RNA from +/- and tg/tg BMMCs was hybridized to dot blots containing rmIL-3, sensitized with 1 μg/ml anti-DNP IgE for 2 h, washed, and then elicited with various concentrations of DNP-HSA. The concentration of β-hexosaminidase in the supernatants and the cell pellets was measured 30 min after elicitation. The percent release of β-hexosaminidase is shown. The values represent the mean ± S.E. of five experiments.

Luciferase Assay—The DNA fragment containing the promoter and 5'-untranslated region of the hPGDS gene (nt -1500 to +200, where +1 is a transcription initiation site) was amplified by PCR from genomic DNA of +/- BMMCs with LA-Taq DNA polymerase (Takara, Kyoto, Japan). This fragment was cloned upstream of the luciferase gene, and the reporter plasmid was constructed. Reporter plasmids sequentially deleted in the promoter region or mutated at the CACCTG motif were also constructed by PCR. The sequence of all constructs was verified using an ABI 3100 sequencer (Applied Biosystems). 10 μg of a reporter, 1 μg of a pEF-BOS expression vector containing MITF cDNA or containing no insert, and 1 μg of an expression vector containing the β-galactosidase gene were cotransfected into BMMCs that had not been elicited were also measured. The values represent the mean ± S.E. of three experiments. In some cases, the S.E. was too small to be shown by bars. * p < .01 by t test when compared with the amount in non-elicited BMMCs.

The DNA fragment containing the promoter and 5'-untranslated region of the hPGDS gene (nt -1500 to +200, where +1 is a transcription initiation site) was amplified by PCR from genomic DNA of +/- BMMCs with LA-Taq DNA polymerase (Takara, Kyoto, Japan). This fragment was cloned upstream of the luciferase gene, and the reporter plasmid was constructed. Reporter plasmids sequentially deleted in the promoter region or mutated at the CACCTG motif were also constructed by PCR. The sequence of all constructs was verified using an ABI 3100 sequencer (Applied Biosystems). 10 μg of a reporter, 1 μg of a pEF-BOS expression vector containing MITF cDNA or containing no insert, and 1 μg of an expression vector containing the β-galactosidase gene were cotransfected into BMMCs by electroporation (350 V, 500 μF). In each transfection, 5 × 10⁶ BMMCs were used. The cells were harvested 48 h after transfection, and the soluble extracts were assayed for luciferase and β-galactosidase activity. The normalized value by β-galactosidase activity was expressed as relative luciferase activity. The effect of MITF on the reporter plasmid was demonstrated by the ratio of the relative luciferase activity with MITF to the relative luciferase activity without MITF (-fold activation).

Electrophoretic Gel Mobility Shift Assay—Production of the fusion protein containing glutathione S-transferase (GST) and MITF was as previously described (23). To examine whether MITF bound to the CACCTG motif mediating the transactivation ability, an oligonucleotide containing this motif was used as a probe. The sequence of the

![Fig. 1. Effect of the concentration of antigen on β-hexosaminidase release in +/- and tg/tg BMMCs. BMMCs derived from +/- and tg/tg mice were established in rmIL-3, sensitized with 1 μg/ml anti-DNP IgE for 2 h, washed, and then elicited with various concentrations of DNP-HSA. The concentration of β-hexosaminidase in the supernatants and the cell pellets was measured 30 min after elicitation. The percent release of β-hexosaminidase is shown. The values represent the mean ± S.E. of five experiments.](http://www.jbc.org/)
oligonucleotide was 5'-CACACAAGACCTGACTGCGACTT (the CACCTG motif is underlined). The oligonucleotide was labeled with [α-32P]dCTP by filling 5'-overhangs and used as a probe for electrophoretic gel mobility shift assay. DNA binding assays were performed in a 20-μl reaction mixture containing 10 mmol/liter Tris-HCl (pH 8.0), 1 mmol/liter ethylenediaminetetraacetic acid (EDTA), 75 mmol/liter KCl, 1 mmol/liter dithiothreitol, 4% Ficoll type 400, 50 ng of poly(dI-dC), 25 ng of labeled DNA probe, and 3.5 μg of GST-MITF fusion protein. After incubation at 37 °C for 15 min, the reaction mixture was subjected to electrophoresis at 14 volt/cm at 4 °C on a 5% polyacrylamide gel in TBE (90 mmol/liter Tris-HCl, 64.6 mmol/liter boric acid, and 2.5 mmol/liter EDTA, pH 8.3). In some experiments, GST was used as a protein instead of GST-MITF. Non-labeled oligonucleotides containing the CACCTG motif or non-labeled oligonucleotides mutated from CACCTG to CTCCAG were added in a competition assay. GST was used as a protein instead of GST-MITF. Non-labeled oligonucleotides containing the CACCTG motif or non-labeled oligonucleotides mutated from CACCTG to CTCCAG were added in a competition assay.

RESULTS

Effect of MITF on the Response of BMMCs Stimulated with IgE and Antigens—The effect of MITF on the response of BMMCs after stimulation with IgE and antigens had not previously been investigated. We examined this by measuring β-hexosaminidase release from BMMCs of tg/tg mice that effectively lacked MITF (5, 23) in response to IgE and antigens. BMMCs were sensitized with anti-DNP IgE, washed, and elicited by various concentrations of DNP-HSA. The released β-hexosaminidase was measured 30 min after elicitation. Levels of released β-hexosaminidase increased in a dose-dependent manner from 5 to 100 ng/ml DNP-HSA, thereafter reaching a plateau (Fig. 1). The +/+ and tg/tg BMMCs showed comparable patterns of β-hexosaminidase release after stimulation with IgE and antigens (Fig. 1).

Production of Eicosanoids in BMMCs Derived from tg/tg Mice—We next examined the generation of eicosanoids in BMMCs of tg/tg mice. BMMCs sensitized with anti-DNP IgE were elicited with 100 ng/ml DNP-HSA, at which concentration the effect of DNP-HSA reached a plateau (Fig. 1). The concentrations of generated PGD2 and LTC4 (the major synthesized eicosanoids in BMMCs) were measured 30 min after elicitation. The amount of generated PGD2 was significantly lower in tg/tg BMMCs than in +/+ BMMCs (Fig. 2). In contrast, the amount of generated LTC4 was comparable between +/+ and tg/tg BMMCs (Fig. 2). Because the generation of PGD2, but not of LTC4, was defective in tg/tg BMMCs, we subsequently concentrated our study on PGD2 production. We examined the process of PGD2 generation elicited by 100 ng/ml DNP-HSA (Fig. 2). Because there was a possibility that tg/tg BMMCs responded at a higher concentration of antigen, we examined generation of PGD2 at various concentrations of DNP-HSA. At all examined concent-
trations (even 1 μg/ml DNP-HSA), PGD2 generation was barely detectable in tg/tg BMMCs (Fig. 3A).

The time-dependent pattern of PGD2 generation elicited with 100 ng/ml DNP-HSA was compared between +/+ and tg/tg BMMCs. In +/+ BMMCs, PGD2 generation was maximal within the first 15 min, and the amount of generated PGD2 did not change over the next 8 h (Fig. 3B). In contrast, PGD2 generation was barely detectable in tg/tg BMMCs at any time during the period of examination (Fig. 3B).

Delayed PGD2 Production in tg/tg BMMCs—Next, we examined delayed phase of PGD2 generation in tg/tg BMMCs. Murakami et al. (17) reported that a delayed phase of PGD2 generation was hardly detectable in BMMCs cultured in medium containing IL-3 alone. Their observations are consistent with the result shown in Fig. 3B in which BMMCs cultured with IL-3 alone were used. We changed the medium for +/+ and tg/tg BMMCs to one containing IL-10, IL-1β, and KitL, in which +/+ BMMCs had been reported to show delayed phase generation of PGD2 (17–20). After culturing BMMCs in this medium for 2 h with anti-DNP IgE, 100 ng/ml DNP-HSA was added. As previously reported (17), the presence of IL-10, IL-1β, and KitL induced generation of PGD2 in +/+ BMMCs without the need for elicitation by DNP-HSA, although in relatively small amounts (0.52 ± 0.02 ng/10⁶ cells, Fig. 3C). Elicited +/+ BMMCs generated PGD2 during the first 15 min, followed by a gradual increase of PGD2 generation over the next 8 h (Fig. 3C). Such patterns of PGD2 generation were not detected in tg/tg BMMCs (Fig. 3C).

To reveal only the delayed phase of PGD2 generation, the immediate phase was eliminated by preincubation of BMMCs with 1 μg/ml indomethacin for 2 h. This reagent inactivates PGHS-1, which is necessary for the immediate phase of PGD2 generation; the PGD2 synthesized in BMMCs preincubated with this reagent reflects the delayed phase of PGD2 generation (18). BMMCs were preincubated with indomethacin in the presence of IL-10, IL-1β, and KitL, sensitized with anti-DNP IgE, washed, and then elicited with DNP-HSA. The +/+ BMMCs synthesized ~2 ng of PGD2/10⁶ cells 8 h after elicitation with DNP-HSA (Fig. 3D). A similar delayed phase of PGD2 generation was barely detectable in tg/tg BMMCs (Fig. 3D).

Deficient Expression of hPGDS Gene in tg/tg BMMCs—The deficient PGD2 generation observed in both immediate and delayed phases suggests that the expression of some gene(s) related to the synthesis of PGD2 might be defective in tg/tg BMMCs. We examined the expression of genes related to PGD2 synthesis using BMMCs cultured in IL-3. The expression levels of genes participating in eicosanoid generation between +/+ and tg/tg BMMCs were compared using CodeLink UniSet mouse expression bioarrays. BMMCs were examined under the following two conditions, BMMCs sensitized with anti-DNP IgE or BMMCs sensitized with anti-DNP IgE and then elicited with DNP-HSA for 30 min. Under both conditions, the levels of expression of hPGDS mRNA were significantly lower in tg/tg BMMCs than in +/+ BMMCs (Table 1). Other genes had comparable expression levels between +/+ and tg/tg BMMCs under both conditions.

We used real-time RT-PCR to confirm the apparently defective hPGDS expression in tg/tg BMMCs. We also quantified the levels of mRNAs from three genes related to PGD2 generation, RasGRP4, PGHS-1, and PGHS-2. We examined BMMCs under the following four conditions, BMMCs without any stimulation, BMMCs sensitized with anti-DNP IgE, BMMCs sensitized with anti-DNP IgE and then elicited with DNP-HSA for 30 min, or BMMCs sensitized with anti-DNP IgE and then elicited with DNP-HSA for 120 min. Under all conditions examined, the hPGDS gene was expressed in tg/tg BMMCs at levels less than one tenth those observed in +/+ BMMCs (Fig. 4). In contrast, the expression levels of RasGRP4 and PGHS-2 genes were comparable between +/+ and tg/tg BMMCs under all conditions. The expression level of the PGHS-1 gene in tg/tg BMMCs was approximately one third that found in +/+ BMMCs, but this magnitude of reduction was small compared with that observed for the hPGDS gene (Fig. 4).

The expression levels of hPGDS, RasGRP4, and PGHS-1 genes were unchanged under the four experimental conditions, whereas the expression level of the PGHS-2 gene increased after stimulation with anti-DNP IgE (Fig. 4). The PGHS-2 expression level further increased following elicitation by DNP-HSA. The magnitude of change in PGHS-2 expression levels induced by stimulation with IgE and antigens was comparable between +/+ and tg/tg BMMCs (Fig. 4).

Expression of hPGDS, PGHS-1, and PGHS-2 genes was examined in terms of protein concentrations using BMMCs without any stimulation. As in the case of mRNA expression, hPGDS protein was detected in +/+ BMMCs but not in tg/tg BMMCs (Fig. 5). The amount of PGHS-1 protein detected was comparable between +/+ and tg/tg BMMCs, and no PGHS-2 protein was detected in either BMMC type (Fig. 5).

### Table 1

| Transcript and its Locus link ID number | Normalized intensity |
|---------------------------------------|----------------------|
|                                       | IgE                  | IgE + Ag              |
|                                       | +/+                  | tg/tg                 |
|                                       | +/+                  | tg/tg                 |
| hPGDS (54486)                        | 11.6                 | 1.1                   |
| PGF2α synthase (64292)               | 0.4                  | 0.4                   |
| PGF1α synthase (19223)               | 0.1                  | 0.1                   |
| PGHS-1 (19224)                       | 4.0                  | 3.4                   |
| RGSP4 (233046)                       | 2.3                  | 1.8                   |
| 15-Lipoxygenase type 1 (11687)       | 10.5                 | 14.4                  |
| 15-Lipoxygenase type 2 (11688)       | 1.9                  | 1.2                   |
| 12-Lipoxygenase (11684)              | 0.5                  | 0.5                   |
| 12-Lipoxygenase, 12R-type (11686)    | 0.6                  | 1.3                   |
| 12-Lipoxygenase, epidermal type (11685) | 2.0              | 2.1                   |
| LTα3 hydrolase (18993)               | 20.9                 | 30.9                  |
| 5-Lipoxygenase-activating protein (11690) | 223.2           | 238.5                 |
| LTC4 synthase (17001)                | 26.6                 | 33.2                  |
| Thromboxane A synthase 1 (21391)     | 35.5                 | 48.8                  |
| ATP-binding cassette, subfamily C (17250) | 13.1             | 16.7                  |

- a: Transcription showing more than 10-fold difference of expression level between +/+ and tg/tg BMMCs that received the same stimuli (IgE or IgE+Ag).
- b: Expression level of PGHS-2 mRNA was too low to detect in this method.
detected in tg/tg BMMCs during the examined period, peaking 2 h after elicitation (Fig. 6).

Enhancement of hPGDS Transcription by MITF—Because real-time RT-PCR and immunoblotting indicated the steady state amounts of hPGDS mRNA and protein, we examined directly whether transcription of the hPGDS gene was defective in tg/tg BMMCs using a nuclear run-on assay. The transcription rate of the hPGDS gene was significantly higher in +/+ BMMCs than in tg/tg BMMCs (Fig. 7). Then we examined the motif mediating the transactivation effect of MITF. The reporter plasmid containing the promoter region of the hPGDS gene (~1500 to +200) was transfected with or without MITF. Luciferase activity increased ~5-fold when transfected with MITF as compared with transfection without MITF (Fig. 8A). This MITF-related increase in luciferase activity was detected even in the reporter plasmid that contained the promoter region starting at ~200 (Fig. 8A). MITF recognizes a CANNTG (N is any nucleotide) motif. Only one CANNTG motif was present in the promoter region starting at ~200 (CACCTG between ~22 and ~17). To examine whether MITF transactivated the hPGDS promoter via this motif, we constructed a reporter plasmid mutated from CACCTG to CTCCAG (the mutated nucleotides are underlined). The mutated reporter plasmid did not show increased luciferase activity mediated by MITF (Fig. 8A).

The binding of MITF to the CACCTG motif was examined by electrophoretic gel mobility shift assay. When a GST-MITF fusion protein was added to labeled oligonucleotide containing a CACCTG motif, a retarded band was detected (Fig. 8B). This retarded band was not found when GST protein alone was added (Fig. 8B). The intensity of this retarded band was gradually weakened by additions of cold oligonucleotide with the CACCTG motif (10-, 50-, and 100-fold molar excess), but not by adding cold oligonucleotide mutated at the CACCTG motif (Fig. 8B). These results demonstrate the specific binding of MITF to the CACCTG motif.

DISCUSSION

BMMCs generate PGD₂ immediately after stimulation with IgE and antigens. When BMMCs are cultured in a medium containing IL-10, IL-1β, and KitL, the amount of generated PGD₂ increases further up to 8 h after stimulation. These immediate and delayed phases of PGD₂ generation were not observed in BMMCs derived from tg/tg mice, indicating that MITF is essential for both phases of PGD₂ generation. Among the genes related to eicosanoid generation, hPGDS showed significant decreases in expression level in tg/tg BMMCs. The hPGDS protein converts unstable PGH₂ generated by PGHS-1, and PGHS-2 into PGD₂. As detected by real-time PCR, the amount of hPGDS protein was barely detected in tg/tg BMMCs. A nuclear run-on assay revealed that transcription of the hPGDS gene was defective in tg/tg BMMCs. MITF appeared to transactivate the hPGDS promoter by binding to the CACCTG motif. Urade and coworkers (21, 24) reported that mice with a disrupted hPGDS gene were defective in +/+ BMMCs. The hPGDS protein was barely detected in tg/tg BMMCs. A nuclear run-on assay revealed that transcription of the hPGDS gene was defective in tg/tg BMMCs. MITF appeared to transactivate the hPGDS promoter by binding to the CACCTG motif.
hibition of RasGRP4 expression with siRNA in a rat mastocytoma cell line decreases hPGDS protein levels. RasGRP4 appears to act as an upstream regulator for hPGDS transcription. In tg/tg BMMCs, RasGRP4 expression levels were comparable with those observed in +/+ BMMCs. However, we have not examined the expression level of RasGRP4 at the protein level, and the possibility that MITF regulates RasGRP4 protein expression remains. Further analyses using an anti-RasGRP4 antibody are needed to address this possibility. An alternative is that MITF acts downstream of RasGRP4. RasGRP4 acts downstream from KIT, and Fisher and colleagues (26) reported that MITF was also a downstream molecule of KIT. The signal from RasGRP4 may activate MITF and subsequently activate expression of hPGDS. RasGRP4 is known to activate the cyclooxygenase pathway, but not the 5-lipoxygenase pathway (22). MITF also activates the cyclooxygenase pathway alone, which supports the hypothesis that MITF is a downstream molecule of RasGRP4.

Murakami et al. (17, 18) reported that elicitation with IgE and antigens increased the amount of PGHS-2 mRNA in BMMCs. In fact, the addition of IgE to BMMCs increased the expression level of PGHS-2 mRNA, and elicitation with the antigen resulted in a further increase. The magnitude of the increase in PGHS-2 mRNA levels was comparable between +/+ and tg/tg BMMCs. Recently, Inoue et al. (27) reported that a metabolite of PGD2 named 15-deoxy-Δ12, 14-prostaglandin J2 (12, 14)-PGJ2 bound the nuclear peroxisome proliferator-activated receptor (PPAR) γ and that PPARγ negatively regulated the expression of PGHS-2 in macrophage cell lines. Because tg/tg BMMCs defective in PGD2 generation normally enhanced the mRNA level of the PGHS-2 gene after stimulation with IgE and antigens, the negative feedback loop between PGD2 and PGHS-2 observed in macrophage cell lines does not appear to apply to BMMCs.

In contrast to the changes in PGHS-2 mRNA levels, the changes in PGHS-2 protein levels in tg/tg BMMCs differed from those observed for +/+ BMMCs. The PGHS-2 protein was barely detected in +/+ BMMCs during the examined period (for 8 h after elicitation). This was consistent with a previous report by Murakami et al. (17) that +/+ BMMCs cultured in IL-3 did not express PGHS-2 protein. In contrast to the case of +/+ BMMCs, PGHS-2 protein was unexpectedly detected in tg/tg BMMCs, with levels peaking 2 h after elicitation. Expression of hPGDS. RasGRP4 is known to activate the cy-
sion of the PGHS-2 gene might be regulated at a translational level, and the mechanism negatively regulating PGHS-2 translation might be defective in \( tg/tg \) BMMCs. Several prostanoïds, such as PGE\(_2\), are known to augment the induction of PGHS-2 (24). Another possibility is that \( tg/tg \) BMMCs might be more sensitive to such prostanoïds than wild type.

KitL induces the expression of PGHS-2 protein (17). We recently reported that the expression of Kit and the response to KitL were partially impaired in \( tg/tg \) BMMCs (28). However, the addition of KitL induced the expression of PGHS-2 protein to comparable levels in +/- and \( tg/tg \) BMMCs (data not shown). The partially deficient signal from Kit appeared to be sufficient for induction of PGHS-2 protein in \( tg/tg \) BMMCs.

PGD\(_2\) is known to recruit eosinophils (3, 4). Recently we found defective eosinophil recruitment in \( tg/tg \) mice (29), suggesting that MITF may play an important role in recruitment of eosinophils through production of PGD\(_2\). MITF appears to be a key transcription factor regulating the function of mast cells.

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