The microphthalmia-associated transcription factor (Mitf) is critical for mast cell development based on the severe mast cell deficiency seen in Mitf mutant mice. Mitf also is important for the development of melanocytes, osteoclasts, and retinal pigment epithelium. The lineage-restricted phenotypes of Mitf mutations correlate with tissue-restricted expression of Mitf, a feature due in part to the presence of several distinct Mitf isoforms. We report the identification and characterization of a novel mast cell isoform, Mitf-mc. This isoform arises from alternative splicing of a novel 5’-exon onto the common body of the gene and is predicted to encode a unique 43-amino acid sequence at its amino terminus. It is specifically expressed in mast cells. The mast cell isoform functions differently from the melanocyte isoform in its ability to activate cell type-specific Mitf gene targets. Mitf-mc functions only on a mast cell target promoter and fails to activate a melanocyte target promoter despite binding to its E-box element. Moreover, Mitf-mc heterodimerizes with a closely related transcription factor, Tfe3, and dominantly inhibits the ability of Tfe3 to transactivate a melanocyte-specific promoter. These studies identify a new isoform of Mitf with tissue-specific features that may underlie key aspects of the mast cell phenotype of Mitf mutations.

Mast cells are central effectors of allergic and hypersensitivity reactions. There is emerging evidence that demonstrates their role in host immunity against parasites and other bacterial infections (1). Overproliferation of these cells results in a spectrum of disorders ranging from benign mastocytosis to mast cell leukemia. Mast cells are derived from hematopoietic stem cells in the bone marrow and spleen (2, 3). From the bone marrow compartment, they migrate to the peripheral connective and mucosal tissues, where they proliferate and mature.

The microphthalmia-associated transcription factor (Mitf) is a member of the basic helix-loop-helix zinc finger transcription factor family, which is essential for the maturation of a diverse collection of cell types. Mice that harbor mutations in the Mitf gene display severe defects in the development of mast cells, melanocytes, osteoclasts, and retinal pigment epithelium (4–6). Mutations in the human gene for the microphthalmia-associated transcription factor, MITF, result in Waardenburg syndrome type IIA (7) and Tietz syndrome (8, 9), which are autosomal dominant disorders characterized by neurosensory hearing loss and pigmentary defects.

The tissues that depend on Mitf for development express a variety of isoforms of this transcription factor. Thus far, six major isoforms of Mitf have been identified: Mitf-m (melanocyte) (10, 11), Mitf-h (heart) (12, 13), Mitf-a (12), Mitf-b (14), Mitf-c (15), and Mitf-e (16). These isoforms differ in their amino termini and arise from differential splicing of a unique first exon onto common downstream exons. Whereas the isoforms differ at their amino termini, they all share the important functional domains of the protein: the transcription domain, basic domain, helix-loop-helix, and leucine zipper. The genomic structure of the human and mouse Mitf genes has been identified, and the expression of each of these isoforms is controlled by distinct promoters (14, 17). The expression of Mitf-m is restricted to melanocytes, whereas Mitf-a is widely expressed. Mitf-h is highly expressed in heart tissue, and Mitf-e was identified from mast cells. This genomic structure involving multiple isoforms may have arisen for at least two reasons: 1) the organization allows for the appropriate temporal and spatial expression of the gene, and 2) the distinct isoforms may in themselves possess cell-specific functions.

A fascinating observation about Mitf is its essential role for the development of diverse cellular fates. One of the reasons for this property is its highly regulated and restricted expression within these committed cell types. Yet how does Mitf activate the expression of melanocyte-specific genes in melanocytes and mast cell-specific genes in mast cells? Conversely, why does Mitf not activate the expression of mast cell-specific genes in melanocytes and melanocyte-specific genes in mast cells? Other mechanisms that may confer distinct cell-specific functions to Mitf include protein interactions with cell-restricted factors and/or cell specific post-translational modifications, such as phosphorylation. Such events may regulate the specificity of gene target activation within different cell types. A means to produce this specificity could be distinct amino termini of these isoforms, which themselves confer cell-specific properties to the protein.

We now report the identification and characterization of a novel mast cell isoform of Mitf, which we call Mitf-mc. Its amplification of 5’-ends; FCS, fetal calf serum; HA, hemagglutinin; RT-PCR, reverse transcriptase-PCR.

This paper is available on line at http://www.jbc.org
expression appears to be restricted to the mast lineage, suggesting a distinct tissue-restricted promoter that allows for regulated expression within mast cells. In addition, despite sharing identical transactivation, DNA binding, and dimerization motifs with the other isoforms, Mitf-mc selectively transactivates the MMCP6 mast cell gene target promoter in reporter assays; it fails to activate the tyrosinase melanocyte target promoter when expressed either in melanocytes or mast cells. We show that selectivity for target promoters is largely mediated by the unique amino terminus of Mitf-mc. Mitf-mc homodimers bind melanocyte-specific DNA target sequences; thus, its failure to transactivate melanocyte targets is not explained by DNA binding preferences. Last, we examine the interaction of Mitf-mc with another closely related transcription factor, Tfe3, which is expressed in mast cells. Mitf-mc was able to bind to Tfe3 as expected and also dominantly inhibited the ability of Tfe3 to transactivate the tyrosinase promoter.

EXPERIMENTAL PROCEDURES

Plasmids—The expression vector, pEBB melanocyte Mitf, was constructed by PCR amplification of the murine melanocyte Mitf sequence from pBS-Mi (18) with NdeI and Clal restriction sites engineered into the 5’- and 3’-ends of the PCR insert. This PCR product was subcloned into the expression vector, pEF-BOS (19), to create pEBB melanocyte Mitf. pEBB mast cell Mitf was made by PCR amplification of the 5’-ends of the PCR insert. This PCR product was subcloned into the expression vector, pEF-BOS, to create pEBB melanocyte Mitf RACE product and using it to replace the 5’ NdeI/BamHI fragment of pEBB melanocyte Mitf. The Mitf domain chimera constructs were made with two-step PCR (20). All Mitf expression constructs include an HA epitope at the amino terminus of the protein. Full-length murine pEBB Tfe3 was constructed by PCR amplification of the Tfe3 sequence from murine mast cell cDNA using primers with NdeI and Clal restriction sites engineered into the 5’- and 3’-ends. pEBB Tfe3 incorporates a FLAG epitope at its amino terminus. For in vitro transcription and translation of the Mitf isoforms, melanocyte Mitf and mast cell Mitf cDNAs were amplified by PCR from the pEBB Mitf expression plasmids and cloned into pcR 2.1 (Invitrogen). This vector contains a T7 promoter that was used for in vitro translation. All constructs made by PCR were verified by sequencing. The human tyrosinase promoter and tandem E-box promoter constructs have been described previously (18, 21). The mouse tyrosinase promoter (22) spanning nucleotides 267 to +65 and the MMCP6 promoter (23) spanning nucleotides −191 to +26 were PCR-amplified from genomic murine DNA and cloned into pGL2 basic (Promega) upstream of the luciferase reporter.

Cells—The C57 mast cell line was kindly provided by S. Galli and maintained in Dulbecco’s modified Eagle’s medium with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and 5 × 10⁻⁵ M β-mercaptoethanol. NIH 3T3 and B16 melanoma cells were grown in Dulbecco’s modified Eagle’s medium with 10% FCS and 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

A Western blot of cell lysates from a variety of Mitf-expressing tissues was probed with a monoclonal antibody to Mitf. Proteins of different mobilities are detected from heart tissue, a melanoma cell line (B16), a macrophage cell line (RAW), and a mast cell line (C57). A weak band is detected from NIH 3T3 cells.
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FIG. 3. The expression of mast cell Mitf is restricted to mast cells. RT-PCR analysis of cDNA from various Mitf-expressing tissues is shown. 5' primers were designed to specifically amplify the various isoforms. The positions of the primers utilized are shown on the left. Primers for β-actin were used as a control. PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide. The sizes of the PCR products are shown on the right. Expression of the mast cell isoform of Mitf is detected only from the mast cell line and primary mast cells.

10% FCS and 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. RAW cells were also maintained in RPMI with 10% FCS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Bone marrow-derived primary mast cells were obtained by culturing cells from spleen and femoral bone marrow of 4–6-week-old C57/BL6 mice in liquid medium (Dulbecco's modified Eagle's medium with 10% FCS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, 20% v/v WEHI-3 conditioned medium). One half of the media was replaced weekly. After 4–6 weeks of culture, over 95% of the cells were identifiable as mast cells by Wright-Giemsa staining.

Animals—4–6-week-old C57/BL6 mice were obtained from the Jackson Laboratory and maintained at the Dana-Farber Cancer institute in accordance with institutional guidelines.

RT-PCR and 5' RACE—Total RNA was obtained from various tissues with Trizol (Invitrogen) and was used to make cDNA with the SuperScript preamplification system (Invitrogen). The 5' primers were as follows: common, 5'-GTGAGAGGAGGAGAGAAGAAG; mast, 5'-CGGGCTGTAGGACTCATCGTACTC; Tfe3, 5'-GTGCAGACCCACCTGGAAAAC; A form, 5'-GAGAACACCTTAAAGGAAGAG; melanocyte, 5'-ATGCGAGAAGCCGCTAGATA; mast, 5'-AGGGGGACTCTTATTTTGTTAG and 3', respectively. PCR products were resolved on a 1.2% agarose gel. The sizes of the PCR products are shown on the left. Expression of the mast cell isoform of Mitf is detected only from the mast cell line and primary mast cells.

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actions containing 5% glycerol, 100 mM KCl, 10 mM Tris-Cl (pH 7.4), 1 mM dithiothreitol, 3
32P-end-labeled DNA probe. 3–5 μl of translated protein was used in each reaction. For the supershift analysis, 5 μl of anti-Mitf antibody C5 was used, and bovine serum albumin or -tubulin antibody was used as control. The reactions were run on 4.5% PAGE with 0.5 TBE with 0.1% glycerol as buffer. The probe sequences are as follows: M box wild type, AAAGTCAGTCATGTGCTTTTCAGA; M box mutant, AAAGTCAGTCATGTGAAGTGCTTTTCAGA.

Transfections—Transfections of 3T3 cells and B16 were carried out in 24-well plates using FuGENE 6 (Life Technologies, Inc.) according to the manufacturer’s recommendations. 1 × 10^5 cells in 500 μl of serum-containing media/well were transfected with 3 μl of FuGENE and 1 μg of DNA. For HMC-1 cells, cells 1 × 10^6 cells in 800 μl of serum-containing media were transfected in 24-well plates using Superfect (Qiagen) according to the manufacturer’s recommendations. 8 μl of Superfect with 4 μg of DNA were used. For transfections of the Mitf expression constructs with the tandem E-box, tyrosinase, and MMCP6 promoter reporters, the driver/reporter ratio was 1:4, and 0.01 μg of sea pansy luciferase plasmid was co-transfected. For the co-transfections of Tfe3 and Mitf, 0.15 μg of the pEBB Tfe3 was used with 0.7 μg of the human tyrosinase promoter reporter, and 0.02 μg of pEBB Tfe3 was used with the MMCP6 promoter reporter. Lysates were harvested after 24 h in 150 μl of passive lysis buffer (Promega). 20 μl of lysate was used to perform luciferase assays using the dual luciferase system (Promega). All experiments were done in triplicate and normalized to sea pansy luciferase activity. COS transfections were performed using the DEAE-dextran method (25). Briefly, 5 μg of DNA was mixed with a 2.5 mM chloroquine and 0.1% DEAE dextran solution and added to 5 ml of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. This mixture was added to a 10-cm plate of confluent COS cells for about 3–4 h at 37°C. The medium was removed, and cells and 10% Me2SO in phosphate-buffered saline were added to the cells for 2 min at room temperature. This solution was removed from the plates, and fresh

**FIG. 4.** Mast cell Mitf isoforms transactivates mast cell-specific promoters but cannot activate melanocyte-specific promoters. The transactivation potential of the melanocyte and mast cell isoforms are compared using luciferase reporter assays. Melanocyte Mitf and mast cell Mitf expression vectors were co-transfected with the reporter constructs depicted on the left. Melanocyte Mitf activates both melanocyte gene target promoters as well as a mast cell gene target promoter (gray bar). Mast cell Mitf (black bar) activates only the mast cell-specific promoter and not the melanocyte-specific promoters. The tandem E-box promoter construct is composed of the target sequences from the tyrosinase promoter M-box. The experiments were performed in 3T3 cells, melanoma cells (B16), and mast cells (HMC-1), and the difference in transactivation potential is demonstrated in all cell types. The shaded box depicts the E-box. Transfection efficiency was normalized to co-transfected sea pansy luciferase activity. The basal activity on the tandem E-box promoter was significantly higher than the basal activities of the other promoters; the relative luciferase activities of the isoforms on each promoter were normalized to the vector activity.

**FIG. 5.** DNA target sequences for Mitf are cell-specific. DNA binding sites for Mitf in melanocyte and mast cell gene target promoters are compared. The sites required for activation of gene expression in melanocyte genes are stringently defined, consisting of a specific core E-box sequence and conserved flanking nucleotides. However, the sites identified within mast cell target genes are composed of a much more loosely defined E-box with no conserved flanking sequences. MMCP, mouse mast cell protease; NGFR, nerve growth factor receptor; MC1R, -melanocyte-stimulating hormone receptor. MC1R promoter contains five E-boxes, only one of which matches the melanocyte sequence (not listed).
medium was added to the cells. Cell lysates were harvested for Western blot or immunoprecipitation in 2–3 days.

RESULTS

Mast Cells Express a Unique Isoform of Mitf—A Western blot of protein lysates from Mitf-expressing tissues probed with a monoclonal antibody to Mitf reveals proteins of differing mobilities (Fig. 1). Protein detected with this antibody in lysates from the C57 mast cell line runs at 75–80 kDa and appears as 3–4 distinct bands. The mobility and appearance of this protein signal differ from the Mitf protein detected in the B16 melanoma cell line, heart tissue, and the RAW macrophage cell line. The major protein species detected from the melanoma cell line is well characterized and is called Mitf-m (melanocyte). It migrates at 60–65 kDa as a doublet, which represents different phosphorylation states of the protein (21). The protein species detected from the other tissues are not yet well characterized but probably represent different isoforms as well as post-translational modifications such as phosphorylation.

5′ RACE was performed with poly(A)−-selected RNA from the murine mast cell line, C57. Using 3′ prime oligonucleotides to the common region of the Mitf (AP1 and AP2), a single PCR product was obtained, which encoded for a novel 5′ prime sequence. The sequence is predicted to result in a novel 43-amino acid sequence at its amino terminus; it is contiguous with the “B” domain of Mitf. An in-frame stop codon lies upstream of the putative start methionine. The full-length protein is predicted to be 534 amino acids; the carboxyl portion of the protein is identical with that of the melanocyte and other Mitf isoforms (Fig. 2, A and B). Using primers specific for this isoform, PCR products were amplified from primary bone marrow-derived mast cells (C57 BL/6) as well as two other mast cell lines P815 and MC/9 and sequenced (data not shown). The sequence was identical to the RACE product obtained from the C57 cell line.

The RACE product was used to generate expression vectors for the full-length mast cell isoform. A Western blot comparing the COS-transfected and in vitro translated Mitf with endogenous mast cell and melanocyte Mitf protein is shown in Fig. 2C. The mobilities of the COS-transfected proteins appear similar to the respective endogenous proteins detected from the mast
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The expression of the various Mitf isoforms was examined in Mitf-expressing tissues by RT-PCR analysis. A 5′ primer specific for each of the different isoforms was used in conjunction with a 3′ primer that anneals within exon 2, which is common to all of the isoforms. For controls, a 5′ primer that anneals within the common exon 2 and primers to amplify beta actin were used. As shown in Fig. 3, the mast cell isoform was expressed in the C57 mast cell line as well as primary bone marrow-derived mast cells but not from the other Mitf-expressing tissues. In addition, the A and heart isoforms were detected from both the mast cell line and primary bone marrow-derived mast cells. As expected, the melanocyte isoform was restricted to the B16 melanoma cell line. The A form was detected from most Mitf-expressing tissues as previously reported (12), including the RAW macrophage cell line and 3T3 NIH fibroblasts.

Mast Cell Mitf Selectively Transactivates Mast Cell-specific Promoters and Not Melanocyte-specific Promoters—To test whether the mast cell isoform, Mitf-mc, could drive expression of known target genes of Mitf, transient transfection assays were performed. As seen in Fig. 4, the melanocyte isoform was able to drive the expression of luciferase under the control of melanocyte Mitf target gene promoters (human and mouse tyrosinase), a tandem E-box-containing artificial promoter (composed of target sequences derived from the tyrosinase promoter M-box), as well as a mast cell target gene promoter (MMCP6). In contrast, the mast cell isoform was able only to transactivate the mast cell target gene promoter. Of note is that the tandem E-box resulted in extremely high basal activity, and the fold activation observed with the melanocyte isoform varied with the amounts and ratios of driver and reporter used; however, the mast cell isoform consistently resulted in activity significantly lower than baseline. Moreover, this selective difference in transactivation potential was not dependent on cell type context, since it was similarly observed in NIH 3T3 cells, a melanoma cell line, B16, and the mast cell line, HMC-1.

The Mitf DNA binding elements found within the melanocyte-restricted target genes and mast cell-restricted targets were compared (Fig. 5). In general, Mitf and other basic helix-loop-helix leucine transcription factors recognize the hexameric E-box sequence defined as CANNTG. Mitf was initially found to recognize an 11-base pair sequence within the melanocyte promoters, tyrosinase and tyrosinase-related protein-1 that consisted of a core E-box, CATGTG. This element was called the “M-box” (22, 26, 27). The DNA sequences in these melanocyte-restricted promoters required for Mitf recognition have recently been more stringently defined, and are composed of the hexameric core, CATGTG, flanked by a highly conserved 5′ T and/or 3′ A (28). Mast cell promoter recognition sites, however, differ. The stringently defined melanocyte binding sites...
are rarely found within Mitf mast cell target gene promoters (23, 29–34). The single known exception is the α-melanocyte-stimulating hormone receptor (35). Whereas this promoter contains five potential Mitf binding sites, only one conforms to the melanocyte recognition sequence. Thus, the promoter elements through which Mitf activates gene expression appear to be distinct between these different cell types.

Next, in order to determine the effects of the unique mast cell amino terminus on transactivation potential, we made a series of constructs in which the amino-terminal domains of the melanocyte and mast cell isoform were interchanged or deleted (Fig. 6A). The melanocyte isoform contains a unique amino-terminal 11 amino acids, whereas the mast cell isoform contains the “B” domain, which is expressed in other isoforms, as well as the unique mast cell amino terminus (Fig. 2B). As can be seen in Fig. 6A, the melanocyte amino terminus appeared dispensable for activation on a melanocytic tyrosinase promoter (white bars). However, the mast cell unique amino terminus was sufficient to confer loss of activation potential (mast/common). The B domain also appeared to confer loss of activation potential but not to the degree of the mast cell domain. This effect of the mast cell amino terminus was observed only on a melanocyte promoter, since the mast cell domain-containing constructs were capable of transactivating a mast cell promoter, MMCP6 (gray bars, basal activity denoted by a dotted line). Comparable expression of these various forms in 3T3 cells is shown in Fig. 6B.

**Mast Cell Mitf Binds the Melanocyte-specific DNA Elements**—One possible mechanism to explain these differences in the transactivation potentials of these isoforms is selectivity for the cell-specific DNA-binding elements. We performed electrophoretic mobility shift assays to address this question. Melanocyte and mast cell Mitf proteins were translated in vitro, and their ability to bind the M-box was assayed in a gel shift experiment. Because of the presence of a strong background band in the reticulocyte lysates, a supershifting monoclonal antibody to Mitf, C5, was used. As can be seen in Fig. 7A, both melanocyte and mast cell Mitf were able to bind the M box as demonstrated by the specific band supershifted by the anti-Mitf antibody. This binding appeared to be specific, since this supershifted band was competed with cold wild type probe but not mutant probe (Fig. 7, B and C). As expected, both melanocyte and mast cell Mitf were capable of binding E box elements since the helix-loop-helix leucine zipper dimerization domain is dispensable for activation on a melanocytic tyrosinase promoter (36) and was used to construct a full-length Tfe3 expression vector. To ask whether the mast cell isoform of Mitf associates with Tfe3, either isoform is capable of being detected in the complex. Conversely, lysates immunoprecipitated with anti-hemagglutinin antibody for Mitf also pull down Tfe3 (Fig. 10B). Thus, Tfe3 appears to associate with both the mast cell and melanocyte isoforms, as predicted, since the helix-loop-helix leucine zipper dimerization domain is remote from the isoform-specific amino-terminal sequences. To
determine whether this was a functional interaction, the ability of Tfe3 and Mitf to transactivate a melanocyte promoter was determined. As seen in Fig. 11A, Tfe3 alone was capable of driving transcription from the melanocytic tyrosinase promoter; however, this activity was dominantly inhibited by increasing amounts of mast cell Mitf. This inhibition by mast cell Mitf on Tfe3 was specific to the melanocyte promoter and not seen on the MMCP6 promoter (Fig. 11B). These results indicate that the novel mast cell isoform of Mitf can selectively prevent activation of a melanocytic promoter even in the presence of other transactivators.
We have identified a novel mast cell isoform of the microphthalmia-associated transcription factor, which we call Mitf-mc. This gene is predicted to encode a unique amino terminus of the protein. The amino-terminal sequence does not resemble any known protein motifs. As expected, the mast cell isoform activates transcription of a mast cell Mitf target gene in reporter assays. Unexpectedly, however, this isoform could not transactivate the melanocyte-specific tyrosinase promoter despite being capable of binding the DNA elements in the promoter. This observation suggests that the mechanism of selectivity for gene target activation is independent of DNA binding preferences and thus is probably due to differential recruitment of protein complexes to the amino terminus of mast cell Mitf. Since this differential effect is seen when a variety of cell types are studied (including melanocytes), the interacting protein(s) that mediate this selectivity are probably ubiquitous.

We examined the interaction of the Mitf isoforms with Tfε3, a transcription factor closely related to Mitf, which is also expressed in mast cells and melanocytes. There was no difference in the ability of the melanocyte and mast cell isoforms to heterodimerize with Tfε3. However, in a functional assay, the mast cell Mitf isoform dominantly inhibited the transactivation activity of Tfε3 on a melanocyte-specific target promoter.

These findings suggest several potential mechanisms that might account for the tissue-restricted action of mast cell Mitf. In chimeras, the mast cell amino terminus represses the transactivation potential of “core” Mitf for the tyrosinase but not the MMCP6 promoter. This suggests that transactivation of these two promoters by Mitf proceeds via distinct biochemical mechanisms that are harnessed by the mast cell isofrom to permit tissue-restricted expression from one gene locus. One possible mechanism to account for these observations is that the mast cell amino terminus might recruit a factor that represses certain promoters (such as tyrosinase) but not others (MMCP6). One argument against this possibility is that the mast cell isoform generally exhibited failure to transactivate rather than true repression (below basal activity). Alternatively, the mast cell amino terminus might intramolecularly prevent association with direct mediators of transactivation, such as p300/cAMP-response element-binding protein-binding protein. Such a mechanism would prevent activation without necessarily triggering true repression (other than by displacement of promoter elements from transactivating isoforms). The dominant inhibition of Tfε3 may come about either through competition by homodimers for the target promoter element or through heterodimers in which the Tfε3 monomer is insufficient to provide a full activation signal. Since Mitf is of major importance in the development of distinct cellular lineages, the identification of tissue-restricted activities by distinct isoforms is likely to provide key insights pertinent to developmental decisions. Further analysis of the mechanistic basis of this activity will thus be of importance.

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