Genetic and Physical Interactions between Microphthalmia Transcription Factor and PU.1 Are Necessary for Osteoclast Gene Expression and Differentiation*

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The microphthalmia transcription factor (MITF), a basic-helix-loop-helix zipper factor, regulates distinct target genes in several cell types. We hypothesized that interaction with the Ets family factor PU.1, whose expression is limited to hematopoietic cells, might be necessary for activation of target genes like tartrate-resistant acid phosphatase (TRAP) in osteoclasts. Several lines of evidence were consistent with this model. The combination of MITF and PU.1 synergistically activated the TRAP promoter in transient assays. This activation was dependent on intact binding sites for both factors in the TRAP promoter. MITF and PU.1 physically interacted when coexpressed in COS cells or in vitro when purified recombinant proteins were studied. The minimal regions of MITF and PU.1 required for the interaction were the basic-helix-loop-helix zipper domain and the Ets DNA binding domain, respectively. Significantly, mice heterozygous for both the mutant mi allele and a PU.1 null allele developed osteopetrosis early in life which resolved with age. The size and number of osteoclasts were not altered in the double heterozygous mutant mice, indicating that the defect lies in mature osteoclast function. Taken in total, the results afford an example of how lineage-specific gene regulation can be achieved by the combinatorial action of two broadly expressed transcription factors.

The MITF gene encodes a basic-helix-loop-helix zipper (bHLH-zip) protein highly related to the TFE3, TFEB, and TFE4 protein family. MITF has been shown to regulate distinct target genes in melanocytes, pigmented retinal epithelial cells, mast cells, and osteoclasts (9–11). MITF can selectively affect gene expression and differentiation of developmentally unrelated types of cells. A major interest of our laboratories is in understanding how MITF selectively activates target genes in osteoclasts as opposed to other cell types where the factor is expressed.

Osteoclasts differentiate from a myeloid progenitor to become mature, multinuclear cells capable of resorbing bone (12). Mice homozygous for the mutant mi allele develop severe osteopetrosis caused by a failure of mononuclear precursors to mature into multinuclear osteoclasts capable of bone resorption (13, 14). Bone marrow transplantation experiments demonstrate that the mi mutation acts in a cell-autonomous manner (15, 16), and in situ hybridization studies confirm that MITF is expressed in osteoclasts beginning at the earliest stages of endochondrial ossification of long bones (17). Understanding how MITF regulates gene expression in osteoclasts may provide insights into the molecular mechanisms that control terminal differentiation of this specialized cell type.

MITF and the related TFE factors form heterodimers and homodimers that bind to DNA sequences related to the E-box motif (CANNTG) shared by many helix-loop-helix transcription factors. In cell types affected by MITF mutations, MITF acts by binding to a cis-acting element, TCATGTG, located in the proximal promoters of lineage-specific genes. For example, genes encoding tyrosinase (melanocytes) and tartrate-resistant acid phosphatase (TRAP) (osteoclasts) require this cis-element for cell type-specific regulation (17, 18). These data indicate that MITF expression alone is likely insufficient to account for the regulation of selective targets in the different cell types. One hypothesis to account for MITF action is that in each cell type, MITF acts in concert with a unique combination of transcription factors to affect expression of target genes.

Expression of the Ets family transcription factor PU.1 distinguishes osteoclasts from melanocytes. PU.1 expression is limited to hematopoietic lineages and is necessary for differentiation of a number of cell types, in particular B-cells, macrophages, and osteoclasts (19, 20). Osteoclast differentiation fails at a very early stage in mice bearing a targeted disruption of the PU.1 gene, but PU.1 is expressed at all stages of osteoclast differentiation in wild-type cells, including in mature multinuclear cells (21). In addition, direct interaction between MITF and PU.1 in vitro has been detected (22).

In this report we present molecular, biochemical, and genetic evidence showing that PU.1 and MITF physically and functionally interact in osteoclasts.
ally interact. We conclude that the interaction with PU.1 is necessary for MITF to affect osteoclast differentiation and gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—Cells were maintained in Dulbecco's modified Eagle's medium containing 4 m\(\text{g}\)l \text{-}glutamine, 50 \(\mu\)g/ml penicillin, 50 \(\mu\)g/ml streptomycin, and 5% newborn calf serum (NIH 3T3 and COS-7) or 10% fetal bovine serum (RAW264.7) at 37 °C in 7% CO\(_2\). Differentiation of RAW264.7 cells was optimized using Dulbecco's modified Eagle's medium (DMEM) supplemented with 50 \(\mu\)M ascorbic acid (Sigma), 20 \(\mu\)g/ml soluble recombinant human RANKL/osteoclast differentiation factor (PeproTech, Inc.), and 10 \(\mu\)M 1,25-dihydroxyvitamin D\(_3\). Cells were seeded at 2 × 10^6 cells in 10-cm dishes 24 h before the transfections. Cells were transfected with Superfect reagent as described by the manufacturer. Cells were harvested 24 h after transfection. RAW264.7 cells were transfected by electroporation as described previously (24).

Expression vectors for PU.1 and MITF and the TRAP luciferase reporter have been described previously (17, 25). Site-directed point mutations of PU.1 and MITF binding sites, indicated in Fig. 1, were introduced by PCR (17). The S36T enhanced green fluorescent protein (GFP) reporter gene-derived pEFGP-1 (CLONTECH) was fused to the TRAP proximal promoter (from −620 to +3, relative to the ATG codon). Under low light conditions, cells were suspended in phosphate-buffered saline and incubated for 10 min at room temperature. Resuspended cells were analyzed for GFP expression using a FACSCalibur flow cytometer (Becton Dickinson). Data analysis was performed by analyzing 10,000 events for each assay using the CELLquest software package (Becton Dickinson). The fluorescence intensity was divided into four regions, M1, M2, M3, and M4. The S36T mutation enhanced GFP expression (100-fold, 1,000-fold, and 10,000-fold above background, respectively). All events with fluorescence intensity greater than the M1 region were accepted as cellular GFP fluorescence events. The data are presented as follows. For TRAP histochemical staining newborn femurs were embedded in plastic resin using a JB-4 embedding kit (Polysciences, Inc.) as described by the manufacturer, and 5-μm sections were cut using the LKB Ultratome (LKB Instruments Inc., Rockville, MD). Samples were stained for TRAP activity using a leucoycte acid phosphatase kit (Sigma) as described by the manufacturer.

**RESULTS**

**PU.1 Regulates the TRAP Promoter through a Conserved GGA Binding Site**—The proximal promoter of the mouse TRAP gene contains a conserved binding site for MITF which is necessary for the transcription of the gene during terminal osteoclast differentiation (17). A putative binding site for the Ets family transcription factor PU.1 (GGA) is located around 10 base pairs upstream of the MITF binding site in the human, mouse, and pig TRAP genes (Fig. 1A). EMSAs were performed with this site using purified recombinant PU.1 (Fig. 1B). These experiments demonstrated that PU.1 specifically recognized the TRAP promoter probe containing the wild-type binding site, but not a probe with a mutated binding site (Fig. 1B, lanes 1 and 2). Additionally, PU.1 binding was competed efficiently by increasing amounts of unlabeled TRAP competitor DNA representing the wild-type binding site, but not the mutant site (Fig. 1B, lanes 3–8 versus 9–14).

To confirm that this site is bound by native PU.1, we examined RAW264.7 macrophage nuclear extracts by EMSA (Fig. 1C). PU.1 is the only macrophage nuclear protein that recognizes the high affinity PU.1 site from SV40 (25). When the TRAP sequence was used in EMSA a complex was detected which comigrated with the SV40 PU-box complex (Fig. 1C, lanes 1 and 2). Addition of anti-PU.1 polyclonal antibody, but not preimmune serum, could block formation of this complex (Fig. 1C, lane 4 versus lane 3). By comparison with the SV40 PU-box, the TRAP site was a substantially lower affinity site as judged by relative cold competition (Fig. 1C, lanes 6–9 compared with lanes 10–13).

The ability of PU.1 to transactivate the TRAP promoter through this binding site was examined using transient transfection assays (Fig. 2A). In NIH 3T3 cells, wild type (WT) PU.1, cotransfection of a PU.1 expression vector was able to activate the TRAP promoter by 4-fold (Fig. 2A, left panel). When the conserved binding site was mutated, activation by PU.1 was abrogated (Fig. 2A, left panel). Similar results were seen when transient assays were performed in RAW264.7 cells that express endogenous PU.1, and basal activity of the TRAP
promoter was decreased about 2-fold in this cell type as well (Fig. 2A, right panel).

Incubation of RAW264.7 cells with colony-stimulating factor-1 and RANKL can promote the differentiation of osteoclast-like cells (29). We used this in vitro differentiation system to examine the role of the conserved PU.1 binding site in regulat-

**Fig. 1.** PU.1 binds to the conserved GGAA sequence on the TRAP promoter. *Panel A,* sequences of the TRAP promoter from mouse, human, and pig contain a conserved PU.1 binding site (GGAA) upstream of the conserved E-box (CACATG). The mutations introduced into the PU.1 binding site and the E-box are shown in small type above the sequences. *Panel B,* 100 ng of the recombinant His$_6$-thioredoxin-PU.1 fusion protein was incubated with $^{32}$P end-labeled wild-type (T) or mutant (GGAA to TTAA, $\Delta$) TRAP promoter. The formation of the complex was competed with increasing amounts (2-, 5-, 10-, 50-, 100-, 200-fold molar excess, lanes 3–8, respectively) of cold wild-type probe (TRAP$\Delta$Pu, lanes 9–14). The arrow indicates the PU.1-TRAP oligonucleotide complex. *Panel C,* RAW264.7 nuclear extracts were incubated with $^{32}$P end-labeled TRAP (T) or SV40 enhancer (S) oligonucleotides. The effect of adding preimmune rabbit serum (lane 3) or anti-PU.1 antiserum (lane 4) to PU.1-TRAP complex formation is shown. Note that this antibody blocks complex formation rather than causing a band supershift (25). The formation of the SV40-PU.1 complex was competed with increasing amounts of the cold SV40 enhancer probe (SV40PU, 0, 5-, 25-, 50-, and 100-fold molar excess, lanes 5–9, respectively) or cold TRAP promoter probe (TRAP, same amounts of competitor, lanes 10–13). The arrow shows the PU.1-containing complex.

**Fig. 2.** PU.1 transactivates the TRAP proximal promoter. *Panel A,* NIH 3T3 or RAW264.7 cells (as indicated) were transfected with 5 µg of either TRAP wild-type (WT) luciferase reporter or the TRAP promoter with the mutated PU.1 site (GGAA to TTAA, $\Delta$Pu.1). The promoter-reporter constructs were transfected either alone ($-PU.1$, 0.5 µg or 2 µg of empty expression vector for 3T3 or RAW264.7, respectively) or together with PU.1-expressing vector (+PU.1, 0.5 µg or 2 µg of expression vector for 3T3 or RAW264.7, respectively). Promoter activity was expressed as relative luciferase units relative to the wild-type basal promoter activity (set to a value of 1). The results of four independent experiments performed in duplicate are represented, and the error bars indicate the standard deviation. *Panel B,* RAW264.7 cells were stably transfected with the indicated wild-type and mutated TRAP reporter constructs (either PU.1 or E-box sites mutated), and pooled clones were grown without RANKL or in the presence of RANKL for 5 days, as indicated. The GFP plasmid without promoter was included as a control. GFP expression was determined by flow cytometry (see “Experimental Procedures”). The graph represents the percentage of cells exhibiting high fluorescence ($10^3$–$10^4$ higher than background fluorescence) among all cells with GFP fluorescence. The average of three experiments is shown with error bars indicating the standard deviation.
MITF and PU.1 collaborate to activate the TRAP promoter. NIH 3T3 cells were transfected with the luciferase reporter (5 µg) driven by either the wild-type TRAP promoter (WT), or the TRAP promoter with mutations either in the E-box (CACATG to CTGGAG, ΔE) or in the PU.1 binding site (GGAA to TTAA, ΔPU). The promoter-reporter constructs were transfected either alone (with empty expression vector) or together with vectors expressing 0.5 µg of PU.1, 3 µg of MITF, or the combination of 0.5 µg of PU.1 and 3 µg of MITF. Promoter activity was expressed as the relative luciferase units with basal activity set to 1 as above. The results of four independent experiments, each performed in duplicate, are presented, with error bars indicating the standard deviation.

MITF and PU.1 cooperate in activation of TRAP reporter genes. To test the hypothesis that interaction with PU.1 might account for the ability of MITF to regulate gene expression in osteoclasts, the combined ability of the two factors to activate the TRAP promoter was studied using transient transfection assays. Expression vectors for PU.1 or MITF alone activated the TRAP reporter 4–5-fold, but the combination of the two factors activated the TRAP reporter 20-fold (Fig. 3). When either the E-box or the PU.1 binding site in the TRAP promoter was mutated, the combination of MITF and PU.1 failed to superactivate the reporter (Fig. 3). The MITF/mi mutation, which encodes a protein lacking the ability to bind to the conserved TRAP E-box (17), failed to superactivate the TRAP reporter in combination with PU.1 (Fig. 3). These results imply a functional interaction between MITF and PU.1 which is dependent on DNA binding of both transcription factors to the TRAP promoter.

Physical interactions between MITF and PU.1. Sato and co-workers (22) have demonstrated previously that MITF and PU.1 can physically interact in vitro when bound to DNA. To confirm and extend these results, biochemical assays were used to study the physical interaction of the two factors in the absence of DNA (Figs. 4 and 5). Coimmunoprecipitation of PU.1 and MITF in COS-7 cells was studied (Fig. 4A). Expression vectors encoding HA-tagged PU.1 and wild-type MITF were cotransfected into COS-7 cells. HA-PU.1 was immunoprecipitated from cells metabolically labeled with [35S]methionine, and the immunoprecipitates were examined by denaturing gel electrophoresis. The experiments demonstrated that a protein with the same mobility as MITF was coprecipitated with PU.1 (Fig. 4A, arrow). This protein was absent from precipitates obtained either from cells expressing only HA-PU.1 (Fig. 4A) or in mock-transfected cells that expressed neither protein (data not shown). In addition, if preimmune serum was used, or if the primary antibody was not included in the immunoprecipitation reaction, neither MITF nor PU.1 was detected (data not shown).

In a complementary set of experiments, full-length recombinant proteins were expressed in Escherichia coli. His6-tagged MITF and GST-PU.1 were separately expressed and purified. GST-PU.1 bound to glutathione-Sepharose beads was used in “pull-down” assays with His6-tagged MITF (Fig. 4B). These experiments demonstrated that His6-tagged MITF could bind to GST-PU.1 Sepharose beads but not to GST-alone beads (Fig. 4B).

To map the domain of MITF involved in the interaction with PU.1, a series of MITF deletion mutations fused to GST was tested in pull-down assays using His6-thioredoxin-tagged PU.1 (Fig. 5). The fragment of MITF containing amino acids 199–298, which included the bHLH-zip domains (Fig. 5A), was able to interact with PU.1 as efficiently as the full-length protein (Fig. 5C, top panel). The protein encoded by the MITF/mi allele was also bound as efficiently as wild-type protein (Fig. 5C, top panel, last two lanes). Neither the N-terminal region including the basic domain (amino acids 1–217) nor the C-terminal portion that included the HLH-zip domain (amino acids 217–419) was able to bind PU.1 above the background level observed with GST alone (Fig. 5C, top panel). In control experiments the MITF deletion proteins did not bind to His6-thioredoxin alone (Fig. 5C, bottom panel). Therefore, both basic and HLH-zip domains of MITF are necessary for efficient PU.1 binding.
Genetic Interactions between MITF and PU.1

PU.1 is a 272-amino acid protein that consists of the N-terminal activation domain, PEST domain, and C-terminal DNA binding domain (Fig. 5B; 20). To map the MITF interacting domain of PU.1 a series of deletions fused to His<sub>6</sub>-thioredoxin were constructed and tested in pull-down binding assays using GST-MITF (amino acids 199–298) as bait (Fig. 5D). Recombinant PU.1 proteins lacking either the activation domain (Δ33–100) or the PEST domain (ΔPEST) were able to interact with MITF, but the protein without the DNA binding domain (Δ201–272) lost the ability to bind MITF (Fig. 5D).

To confirm the minimal regions of MITF and PU.1 required for the interaction, the in vitro binding assay was performed using amino acids 199–298 of MITF fused to GST (Fig. 5A) and amino acids 161–272 of PU.1 fused to His<sub>6</sub>-thioredoxin (Fig. 5B). These experiments demonstrated that the DNA binding domain of PU.1 was able to bind GST-MITF 199–298 fusion protein, but not GST (Fig. 5E). Inclusion of ethidium bromide in these reactions had no effect on the pull-down results, consistent with the conclusion that interaction between free proteins in solution, and not protein bacterial DNA complexes, is being measured in these assays (data not shown).

**Fig. 5. Identification of MITF and PU.1 interacting domains.** Panel A, diagram of MITF, indicating protein domains expressed as GST fusion proteins in *E. coli*. The position of the *mi* mutation, a deletion of one of four critical arginine residues in the basic region, is indicated (arrow). LZ, leucine zipper. Panel B, diagram of PU.1 indicating protein domains expressed as His<sub>6</sub>-thioredoxin fusion proteins in *E. coli*. In panels C–E, the binding reactions with recombinant proteins were incubated with glutathione-Sepharose beads (or Sepharose beads alone) and washed; bound material was eluted and resolved on 10% SDS-PAGE and analyzed by Western blotting using antibodies against His<sub>6</sub> tag (Santa Cruz Biotechnology). **DBD**, DNA binding domain; **AD**, activation domain. Panel C, 1 μg of recombinant His<sub>6</sub>-thioredoxin-PU.1 (PU.1) or His<sub>6</sub>-thioredoxin (Trx) was incubated with 1 μg of recombinant GST or GST-MITF fusion proteins, as indicated. MITF-mi represents full-length MITF protein with the 3-base deletion that defines the *mi* allele. Panel D, 1 μg of recombinant PU.1 or PU.1 deletion mutants (Fig. 5B) fused to His<sub>6</sub>-thioredoxin or His<sub>6</sub>-thioredoxin alone (Trx) was incubated with 1 μg of recombinant GST or MITF bHLH-zip fused to GST (amino acids 199–298). Panel E, 1 μg of PU.1 DNA binding domain (amino acids 161–272) fused to His<sub>6</sub>-thioredoxin or His<sub>6</sub>-thioredoxin alone (Trx) was incubated with 1 μg of recombinant GST or GST fused to the bHLH-zip domain of MITF (amino acids 199–298).

Mutations of the binding site for either transcription factor abolished the formation of the complex containing both MITF and PU.1 (Fig. 6A). Cold competition experiments to measure off time rates of the complexes were also performed (Fig. 6B). Consistent with the data presented above, the PU.1 complex had a very rapid off rate of less than 30 s. The MITF complex was very stable, with little competition observed even after 30 min. The complex containing both factors behaved the same as the PU.1 complex, with a very rapid off rate (Fig. 6B). Taken together, the data indicate that the presence of MITF, which binds stably and with high affinity, does not alter the low affinity binding of PU.1 to the adjacent site.

**Genetic Interaction between MITF and PU.1**—Ultimately, the relevance of interactions between MITF and PU.1 can only be assessed by examining interactions at the genetic level in intact animals, where both the target cell population and the levels of expression of the gene products are appropriate. Mice heterozygous for either the *mi* allele (MITF/ *mi*) or for a PU.1 knockout allele do not develop an apparent bone phenotype. To test for the possibility of genetic interaction between MITF and PU.1 we crossed heterozygous MITF/ *mi* mice with heterozygous PU.1/ + animals to obtain double heterozygous MITF/ *mi* / PU.1 +/− mice. The resulting mice were analyzed for bone density and morphology by radiological techniques and hematoxylin and eosin staining (Figs. 7, A and B, respectively). Approximately 25% of the double heterozygous MITF/ *mi* / PU.1 +/− mice were clearly osteopetrotic compared with 0% of PU.1 +/− and 5% of MITF/ *mi* single heterozygotes, respectively.

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**Panel C**

**Panel D**

**Panel E**

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**Panel C**

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**Panel C**

**Panel D**

**Panel E**
regulation of osteoclast target genes is that MITF might cooperate with a hematopoietic cell-restricted factor in this cell type. Results from both transfection studies and from in vitro biochemical assays support the hypothesis that the Ets family factor PU.1 and MITF act together to regulate the mature osteoclast marker gene, TRAP. Consistent with the biochemical data, combining mutant alleles for MITF and PU.1 in mice demonstrated an interaction between these two genes in vitro during osteoclast differentiation.

Osteopetrosis was detected early in life in mice heterozygous for a PU.1 knock-out allele and the mi allele. The morphology and number of osteoclasts appeared normal in bone sections examined in the compound heterozygous mice, indicating that the defect occurred during the later stages of osteoclast differentiation or in fully mature osteoclasts. The similar phenotypes of mice with targeted deletions of two known MITF target genes, TRAP and cathepsin K, support a role for both MITF and PU.1 in mature osteoclasts (17, 30–32). Multinuclear os-
teoclasts are present in both TRAP and cathepsin K knock-out models, but these osteoclasts are not fully functional, and mild osteosclerotic disease occurs in the mice (31, 32). The inability to regulate expression of these MITF target genes properly could account for the phenotype reported here (17, 30). It is worth noting that the cathepsin K promoter region shown to be resulting from the F2 cross of the unrelated MITF/PU.1 heterozygotes (7, 8). Similarly, other Ets family transcription factors expressed in the macrophage lineage (e.g. Ets-2, ELF-1, MEF, 35, 36), some of which can also trans-activate the TRAP promoter2, could ameliorate the effects of the absence of PU.1. Additional biochemical and genetic analyses will be required to determine the role of these bHLH-ZIP and Ets factors as well as to identify other putative PU.1 and MITF partners in osteoclast differentiation.

The Mechanism of MITF and PU.1 Collaboration—PU.1 interactions with several different transcription factors have been documented (37). For example, PU.1 recruits the transcription factor PIP to DNA through direct physical interactions, an event necessary for activation of target genes in B-cells (38–40). These results led us to anticipate that MITF and PU.1 might bind cooperatively to DNA, but all evidence obtained here indicated that PU.1 and MITF bound independently to TRAP promoter sequences. Because MITF and PU.1 are coexpressed in both macrophages and osteoclasts, a mechanism based on cooperative DNA binding might lead to inappropriate expression of genes like TRAP in macrophages. Further, if the two factors bind to DNA independently, the weak TRAP PU.1 binding site may provide a mechanism by which TRAP promoter activity is sensitive to the increased PU.1 expression levels observed during osteoclast differentiation (21).

Binding sites for both PU.1 and MITF were required for superactivation of the TRAP promoter, indicating that a ternary complex with DNA might be requisite for functional interactions to occur. By contrast, the bHLH factor MyoD and its cofactor for regulation of muscle-specific genes, MEF2, require only a single binding site for either factor to allow synergistic activation of target genes (41). The active ternary complex formed when both factors bind to DNA may result in conformational changes in both proteins which allows for more efficient interaction with transcriptional coactivators. PU.1 and MITF have both been shown to be targets of signaling pathways in other cell types (39, 42, 43). Modification of either MITF or PU.1 by signaling pathways could also enhance the formation of the ternary complex and couple complex formation to signals triggering osteoclast differentiation.

Other mechanisms that depend on PU.1 and MITF interaction prior to DNA binding cannot be ruled out by our data. The overexpression of MITF/PU.1 can lead to retention of PU.1 in the cytoplasm of transfected WEHI-3 cells (22). The formation of a PU.1-MITF complex in the cytoplasm and cotranslocation into the nucleus could ensure that the two factors are recruited together to promoters such as TRAP, effectively increasing the on rate for binding and the likelihood that both sites on the promoter are occupied. Such a mechanism could also be regulated by post-translational modification of either or both factors.

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a A. I. Cassady and D. A. Hume, unpublished data.
In summary, we have shown that PU.1 and MITF cooperate to activate the process of osteoclast differentiation and transcriptional activation of the osteoclast-specific TRAP gene. These findings add to the increasing evidence that unique combinations of widely distributed transcriptional regulators like MITF and PU.1 can generate lineage-restricted gene expression.

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