Induce the Leukocyte Receptor Osteoclast-associated Receptor Microphthalmia Transcription Factor and PU.1 Synergistically Induce the Leukocyte Receptor Osteoclast-associated Receptor Gene Expression*

Received for publication, March 21, 2003
Published, JBC Papers in Press, April 13, 2003, DOI 10.1074/jbc.M302940200

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We have recently reported the identification of a novel member of the leukocyte receptor family, osteoclast-associated receptor (OSCAR), which has two Ig-like domains and functions as a bone-specific regulator of osteoclast differentiation. Here, we have cloned the OSCAR promoter region to examine its regulation by transcription factors. The 1.7-kb promoter region of the mouse OSCAR gene contains two potential E-box elements for microphthalmia transcription factor (MITF) and three putative PU.1 sites. MITF or PU.1 alone activates the OSCAR reporter construct 5–6-fold, and the combination of MITF and PU.1 synergistically activates the OSCAR reporter activity up to 110-fold. The mRNA expression patterns of MITF, PU.1, and OSCAR in TRANCE-treated (RAW 264.7) or TRANCE/M-CSF-treated cells (primary osteoclasts) reveal that MITF mRNA expression is induced at a much earlier time point than OSCAR gene expression. In contrast to MITF, PU.1 mRNA levels remain relatively constant at all time points, suggesting that TRANCE-induced MITF, not PU.1 expression, is one of the critical regulatory mechanisms for optimal OSCAR expression during osteoclastogenesis. In addition, we have shown that the combination of MITF and constitutively active MKK6-expressing plasmids synergistically activates OSCAR reporter activity. Taken together, our results strongly suggest that the activation of MITF and constitutively active MKK6-expressing plasmids synergistically activate OSCAR gene expression. Moreover, the activation of OSCAR gene expression by PU.1/MITF is further enhanced by the TRANCE-induced MKK6/p38 signaling cascade.

Bone is a dynamic tissue that provides mechanical support, physical protection, and a storage site for systemic mineral homeostasis. Bone is continuously remodeled and balanced through the activity of osteoblasts and osteoclasts. Osteoblasts, originating from osteoprogenitor cells, are responsible for the mineralization of bone matrix. Osteoclasts, differentiated from hematopoietic cells, are unique cells that can resorb mineralized bone.

Two essential factors, M-CSF1 and TRANCE, are produced by osteoblasts and can support osteoclast differentiation from monocyte/macrophage lineage cells (1–3). The spontaneous mouse mutant op/op, defective in M-CSF, shows an osteoprotropic phenotype due to the defective differentiation of osteoclasts (4–6). The tumor necrosis factor family member TRANCE (also known as receptor activator of NF-κB ligand (RANKL), osteoclast differentiation factor (ODF), and osteoprotegerin ligand (OPGL)) (2, 3, 7, 8), is expressed on the surface of osteoblasts and is required by osteoclast precursors for their differentiation into mature osteoclasts (9, 10).

In addition to M-CSF and TRANCE, osteotropic factors such as 1,25-dihydroxyvitamin D₃, parathyroid hormone, prostaglandin E₂, and interleukin-11 can stimulate osteoclast formation via up-regulation of TRANCE expression (1, 2). Other cytokines such as interleukin-1, interferon-γ, tumor necrosis factor-α, and transforming growth factor-β can act directly on osteoclast progenitors or on mature osteoclasts (11–17).

The MITF gene encodes a basic helix-loop-helix zipper protein that is important for differentiation of osteoclasts (18). A mutant allele of the MITF gene, m1, encodes a protein lacking one of four arginines in the basic region of MITF protein that is critical for binding to target genes. MITFmutant mice exhibit severe osteopetrosis caused by failure of mononuclear precursors to mature into multinuclear osteoclasts capable of bone resorption (19, 20). It has been shown that MITF regulates expression of the tartrate-resistant acid phosphatase (TRAP) and cathepsin K genes during osteoclast differentiation by binding to E-box-type enhancers in the 5′-flanking regions of MITF-responsive genes (19, 21–23). Moreover, Mansky et al. (24) showed that TRANCE signaling in primary osteoclasts resulted in MITF phosphorylation mediated by a MKK6/p38 signaling cascade that correlated with TRAP expression. These results indicate that MITF is a direct target of TRANCE/p38.
Synergistic Transactivation of OSCAR by MITF and PU.1

DNA was prepared by reverse transcription-PCR-amplified using RNA from primary osteoclasts. The primers are as follows: 5′-ACAG TTC ATG TTA CAG GGG TCC AAA ATG GAA GAA GGG-3′ (5′-PU.1 HindIII) and 5′-ATA AGA ATG CCG CTC AGT GGG GGG GGA GCC GCC-3′ (3′-PU.1 NotI). This amplified PCR fragment was digested with HindIII and NotI and cloned into the pFLAG CMV2 vector. All expression plasmids were confirmed by DNA sequencing. The 1.7-kb OSCAR promoter region (−1862 to +68 bp) was amplified from mouse genomic DNA by PCR using the following primers: 5′-AGAG CCG CCG CAG ACG ACA GTA ATG TCA GGA-3′ (sense) and 5′-CCCT CTC GAG CCG TGG AGC TGA GAA GGA GGA GGT-3′ (antisense). The PCR product was then cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen), digested with NotI and Xhol, and cloned into the pGL2 basic luciferase vector (pGL2 OSCAR). Deletion mutants of the E-boxes, pGL2 OSCAR 1.0 (−906 to +68) were generated by PCR and cloned into pGL2 basic vector. Point mutations of E-box 1 and E-box 2 in the OSCAR promoter were generated by the QuikChange method of site-directed mutagenesis (Stratagene). The oligonucleotides used for site-directed mutagenesis were as follows: E-box 1 point mut, 5′-GGTT TTA ATT TCC GAT AAC ATG ATG TTG CTA CCA TCT GC-3′ (sense) and 5′-GCA GAT GAT GTA ACC ATC TTA TCT CTA GAG AAA CCA-3′ (antisense); E-box 2 point mut, 5′-CCCC TAA ACA GCA CCG TCTA TGGA GGG GGG-3′ (antisense); E-box 2 point mut, 5′-TTT CCT AGT GGT TGT TGA ATT TCT CTG CTT ACC-3′ (sense) and 5′-GGGT GGG AGG AAG AAT GAA AAA GTG TAA ACC AAC AAC-3′ (antisense); and E-box 2 point mut, 5′-TTTT CTT ATT TCTT ATT TCT AAC ACC CAC TCC TTA CCC-3′ (sense) and 5′-GGGA AAG GGG GGG GTAG TTA AGA AAA AAT GGG GAA-3′ (antisense). Constitutively active MKK6 expression plasmid was previously described (24).

Transfection and Luciferase Assay—For transfection of reporter plasmids, 293T cells were plated on 24-well plates at a density of 1 × 10^5 cells/well on 1 day before transfection. A total of 800 ng of plasmid DNA including 100 ng of luciferase reporter, 250 ng of MITF expression vector, 500 ng of PU.1 expression vector, and 10 ng of pcDNA3-β-gal, except as noted, was transfected into 293T cells by calcium phosphate precipitation as described (30). The amount of transfected DNA was held constant to 860 ng by the addition of empty vector DNA where necessary. After 48 h of transfection, the cells were washed twice with phosphate-buffered saline buffer and then lysed in reporter lysis buffer (Promega). Luciferase activity was measured with a luciferase assay kit (Promega) according to the manufacturer’s instructions. For RAW264.7 transfection, cells were suspended in Dulbecco’s modified Eagle medium containing 10% fetal bovine serum, seeded at 5 × 10^6/well in 24-well plates, and cultured for 24 h. Cells were incubated with a total of 2 μg of plasmid, 6 μl of Tfl−50 reagent (Promega), and 200 μl of serum-free medium. After a 1-h incubation, 800 μl of Dulbecco’s modified Eagle medium containing 10% fetal bovine serum was added, and cells were cultured for 48 h. After 48 h of transfection, luciferase activity was measured.

Expression Vectors and OSCAR Promoter Luciferase Constructs—The wild type (MITF-WT) and MITFmutant mutant type (MITF-mi/mi) genes were made by PCR amplification from recombinant adenoviruses (23). The primers are as follows: MITF-WT, 5′-CCC AAG CTT ATG GGG CTC AGA ATG CTA GAA TAT-3′ (sense) and 5′-ATA AGA ATG CCG CTC ACC AAG TGG TCT CGC CTC-3′ (antisense); MITF-mi/mi, 5′-CCC AAG CTT ATG GGG CTC AGA ATG CTA GAA TAT-3′ (sense) and 5′-ATA AGA ATG CCG CTC ACC AAG TGG TCT CGC CTC-3′ (antisense). These fragments were then cloned into the HindIII and NotI sites of the pFLAG CMV2 vector. The murine full-length of PU.1 signaling pathways essential for osteoclast differentiation and function.

The ETS family transcription factor PU.1 is preferentially expressed in the hematopoietic cell lineage and plays a pivotal role in the differentiation of a number of cell types, including B cells, macrophages, and osteoclasts (25, 26). Although PU.1 is expressed at all stages of osteoclast differentiation in wild type cells, osteoclasts from PU.1 knockout mice show a failure to differentiate at a very early stage, eventually leading to an osteopetrotic phenotype. It has also been reported that direct interactions between MITF and PU.1 are necessary for osteoclast gene expression and differentiation (27).

We have recently shown that OSCAR, a novel member of the leukocyte receptor complex-encoded family, is specifically expressed in osteoclasts and regulates osteoclastogenesis (28). Genes in the leukocyte receptor complex family encode different types of Ig-like surface receptors and play critical roles in the regulation of both innate and adaptive immune responses. As opposed to other members of the leukocyte receptor complex family, OSCAR expression has only been detected in preosteoclasts and mature osteoclasts, and it has been suggested that OSCAR may play an important role as a bone-specific regulator (28). Therefore, we examined whether osteoclast-specific OSCAR gene expression is also regulated by both MITF and PU.1.

We report here that OSCAR expression is regulated by the combined activity of MITF and PU.1. For this cooperation, binding of MITF to its cognate sites in the OSCAR promoter is critical, whereas binding of PU.1 to its recognition sites is dispensable. In addition, the induction of the OSCAR gene is further regulated by TRANCE/p38/MITF signaling cascades. Therefore, this study indicates that the expression of a novel osteoclast-specific gene, OSCAR, is regulated by both TRANCE/p38/MITF signaling pathways and a cooperative transactivation between MITF and PU.1.

EXPERIMENTAL PROCEDURES

Reagents—All cell culture media and supplements were obtained from Invitrogen. Soluble recombinant mouse TRANCE was purified from insect cells as described (7), and recombinant human M-CSF was a gift from Dr. David Fremont (Washington University, St. Louis, MO). TRIZOL was purchased from Invitrogen. The Ready-to-Go labeling kit and ProbeQuant G-50 purification kit were from Amersham Biosciences.

Bone Marrow or Spleenocyte-Derived Osteoclast Generation and Total RNA Preparation—Murine osteoclasts were prepared from bone marrow cells or spleenocytes as previously described (28, 29). In brief, bone marrow-derived cells (1 × 10^7/100-mm dish) from C57BL/6 mice (4–6 weeks of age) were incubated with M-CSF (5 ng/ml) in α-minimal essential medium containing 10% fetal bovine serum for 16 h. After incubation, the floating cells were collected and further cultured with M-CSF (30 ng/ml), TRANCE (1 μg/ml), and prostaglandin E2 (10^−6 M) for 4 days. On day 3, media containing M-CSF, TRANCE, and prostaglandin E2 were replaced. On day 4, the cells were washed with phosphate-buffered saline buffer and then harvested. Total RNA was prepared using the TRIZOL reagent according to the manufacturer’s instructions.

Northern Blot Analysis—Total RNA samples were electrophoresed and transferred to nylon membranes as described (7). Hybridization was performed at 42 °C for 16 h with a ^32P-dCTP-labeled DNA probe prepared using the Ready-to-Go labeling kit and ProbeQuant G-50 purification kit. After washing with 0.1 SSC and 0.1% SDS at 56 °C for 1 h, the membrane was exposed to x-ray film.

Expression Vectors and OSCAR Promoter Luciferase Constructs—The wild type (MITF-WT) and MITFmutant mutant type (MITF-mi/mi) genes were made by PCR amplification from recombinant adenoviruses (23). The primers are as follows: MITF-WT, 5′-CCC AAG CTT ATG GGG CTC AGA ATG CTA GAA TAT-3′ (sense) and 5′-ATA AGA ATG CCG CTC ACC AAG TGG TCT CGC CTC-3′ (antisense); MITF-mi/mi, 5′-CCC AAG CTT ATG GGG CTC AGA ATG CTA GAA TAT-3′ (sense) and 5′-ATA AGA ATG CCG CTC ACC AAG TGG TCT CGC CTC-3′ (antisense). These fragments were then cloned into the HindIII and NotI sites of the pFLAG CMV2 vector. The murine full-length of PU.1...
RESULTS

OSCAR mRNA Level Is Decreased in MITF<sup>mi/mi</sup> Osteoclasts—MITF, a member of helix-loop-helix transcription factor family, is abundantly expressed in osteoclasts and can bind to the E-box consensus sequence (CA(C/T)(G/A)TG). Recently, it has been shown that MITF regulates TRAP and cathepsin K expression in osteoclasts (23, 27). To examine OSCAR expression in MITF mutant osteoclasts, mouse osteoclasts were prepared from wild type and MITF<sup>mi/mi</sup> mutant mice. In the wild type culture, giant TRAP(+) multinuclear cells were detected after 7 days of incubation with M-CSF, TRANCE, and prostaglandin E<sub>2</sub>. In the MITF<sup>mi/mi</sup> mutant culture, TRAP activity was much weaker, and TRAP(+) multinuclear cells were much smaller, with only a few nuclei at day 7 (data not shown). Consistently, TRAP, cathepsin K, and OSCAR expression were significantly reduced in MITF<sup>mi/mi</sup> osteoclasts (Fig. 1).

There Are Two E-boxes, the Consensus Binding Sites for a Transcription Factor MITF, and Three PU.1 Boxes, the Consensus Binding Sites for PU.1, in the OSCAR Promoter—Examination of the 1.7-kb mouse OSCAR promoter region revealed that there were no TATA or CAAT box consensus sequences located in the 5' upstream region of the transcriptional start site. However, there were two potential E-box elements at −929 and −6 and three putative PU.1 recognition sites at −1472, −256, and −237 of the 1.7-kb mouse OSCAR promoter fragment (Fig. 2).

OSCAR Is a Target of the MITF Transcription Factor—To examine whether MITF can directly regulate OSCAR expression in osteoclasts, we used a reporter assay involving transient transfections into 293T cells. The 1.7-kb OSCAR promoter region, including the transcription initiation site, was constructed in the pGL2 basic luciferase vector. This pGL2 OSCAR 1.7 plasmid was co-transfected with various amounts of wild type MITF or MITF<sup>mi/mi</sup> mutant-type plasmid. As shown in Fig. 3A, the luciferase activity was increased in a dose-dependent manner by MITF-WT but not by MITF-mi/mi. Because MITF expression increased the OSCAR gene promoter activity, we further examined which of the two E-boxes is critical for MITF binding. To address this question, various deletion plasmids were constructed and transfected into 293T cells. As shown in Fig. 3B, luciferase activities were significantly decreased in the pGL2 OSCAR 0.1 (+2 to +68), and the pGL2 OSCAR 1.0 (+966 to +1626; E-box 2) constructs were compared with the pGL2 OSCAR 1.7 construct (−1626 to +68; E-box 1 and E-box 2). Next, using site-directed mutagenesis, we generated constructs with point mutations within each single E-box 1 and E-box 2, and one double E-box of the 1.7-kb OSCAR promoter fragment. As shown in Fig. 3C, all types of E-box mutations significantly decreased the luciferase activity in the presence of MITF. To further confirm whether MITF directly binds to these E-boxes, EMSA was performed. Endogenous MITF from nuclear extract

Fig. 1. OSCAR mRNA expression in wild type and MITF<sup>mi/mi</sup> osteoclasts. Total RNA was collected from day 6 culture of wild type and MITF<sup>mi/mi</sup> osteoclasts and analyzed by Northern blot using probes for OSCAR, cathepsin K, TRAP, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Fig. 2. Nucleotide sequences of the putative promoter region of mouse OSCAR. The OSCAR promoter contains two potential MITF-binding E-boxes and three potential PU.1 transcription factor recognition sites. The numbers on the left are the nucleotide positions relative to the transcription initiation site.
Synergistic Transactivation of OSCAR by MITF and PU.1

A) the 1.7-kb mouse OSCAR promoter luciferase reporter (pGL2 OSCAR 1.7) was transfected into 293T cells in the presence of various amounts of wild-type and MITFmi/mi expression plasmids. B) OSCAR promoter luciferase reporters, which have different sizes, were transfected into 293T cells in the presence of various amounts of wild-type and MITFmi/mi expression plasmids. C) the 1.7-kb mouse OSCAR promoter luciferase reporters containing wild type or point mutations within the E-boxes were transfected into 293T cells in the presence of various amounts of wild-type and MITFmi/mi expression plasmids. Each well was also co-transfected with 10 ng of a β-galactosidase expression vector to control for transfection efficiency. Luciferase activities were normalized to β-galactosidase activity as expressed by the co-transfected plasmid. The level of the wild type 1.7-kb OSCAR reporter construct in the presence of empty expression vector was set to 1. Results are representative of at least three independent sets of similar experiments (A–C). Data represent the mean and the S.E. of triplicate samples.

of cultured wild-type mouse osteoclasts was found to bind to E-box 1 and 2. The specificity of this binding was confirmed by competition studies using cold wild- and mutant-type competitor probes (Fig. 4, A and B). Results suggest that OSCAR is a target of the transcription factor MITF.

OSCAR Is Also a Target of Transcription Factor PU.1—Recently, it has been reported that genetic and physical interactions between MITF and PU.1 are necessary for osteoclast gene expression and differentiation (22). Furthermore, there are three PU.1 binding sites in the OSCAR 1.7-kb promoter region. Therefore, we examined the effect of the PU.1 transcription factor on OSCAR gene expression. Luciferase activity was increased in a dose-dependent manner by the PU.1 expression plasmid (Fig. 5A). Because PU.1 also increased the OSCAR gene promoter activity, we further examined which potential binding site is important in these promoter activations. To address this question, various constructs containing PU.1 box point mutations (Fig. 5B) were constructed and transfected into 293T cells. As shown in Fig. 5B, all types of PU.1 binding site mutants except the PU.1 box 1 mutant showed significant decreases in the luciferase activity in the presence of the PU.1.

To further confirm whether PU.1 directly binds to its recognition sites, EMSA was performed. The purified GST-PU.1 protein was found to bind to PU.1 recognition sites 2 and 3. Specificity of this binding was confirmed by competition studies using cold wild- and mutant-type competitor probes (Fig. 5, D and E). However, GST-PU.1 protein could not specifically bind to PU.1 recognition site 1 (Fig. 5C). This result is consistent with the inability of mutant PU.1 box 1 to decrease OSCAR gene promoter activity (Fig. 5B). Taken together, these results suggest that OSCAR is also a target of the transcription factor PU.1.

The Combination of MITF and Constitutively Active MKK6 Synergistically Activated the OSCAR Promoter—TRANCE has previously been shown to activate multiple signaling pathways
including NF-κB, AP-1, p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, and Akt via recruitment of tumor necrosis factor receptor-associated factor molecules, which are associated with TRANCE receptor (also known as receptor activator of NF-κB, or RANK) (30, 31). These signaling events are important for osteoclast differentiation and function. In particular, Mansky et al. (24) reported that MITF was rapidly and persistently phosphorylated upon stimulation of primary osteoclasts with TRANCE and that MITF phosphorylation at Ser307 correlated with expression of the TRAP target gene. Therefore, we examined whether this p38 pathway is involved in OSCAR gene expression. To address this question, TRANCE receptor, MITF, and OSCAR 1.7-kb reporter plasmids were co-transfected into RAW264.7 cells and stimulated with various doses of TRANCE for 48 h. Although RAW264.7 cells constitutively express endogenous TRANCE receptor, TRANCE did not induce detectable levels of OSCAR promoter activity in transient transfection assays unless the TRANCE receptor was overexpressed (data not shown). However, luciferase activity was significantly increased by overexpression of the TRANCE receptor and MITF (Fig. 7A). The combination of TRANCE receptor and MITF-expressing plasmids without TRANCE induced an approximately 8-fold increase in OSCAR promoter activity compared with the control (pGL2 basic + pFLAG CMV1 TR + pFLAG CMV2 MITF-WT). However, the combination of TRANCE receptor and MITF-expressing plasmids with 1 μg/ml TRANCE induced an approximately 20-fold increase in OSCAR promoter activity compared with this control.

MKK6 has previously been reported to be an upstream activator of the p38 mitogen-activated protein kinase cascade (32). In addition, Mansky et al. (24) reported that Rac1/MKK6/p38/MITF signaling cascades are involved in TRANCE-induced TRAP gene expression. To address the involvement of the MKK6/p38/MITF signaling cascade in OSCAR gene expression, we tested whether the ability of MITF to transactivate the OSCAR 1.7-kb reporter construct would be enhanced by constitutively active MKK6. Co-transfection of the OSCAR reporter with wild type MITF resulted in approximately a 3-fold increase in promoter activity compared with control. When constitutively active MKK6 was co-transfected with the OSCAR reporter, there was a 0.9-fold increase compared with control basal activity. When MITF and constitutively active MKK6-expressing plasmids were co-transfected with the OSCAR reporter, there was a greater than 14-fold increase in promoter activity relative to the control (Fig. 7B). These data suggest that MITF collaborates with the MKK6/p38/MITF signaling pathway to stimulate OSCAR promoter activity.

The Expression of MITF mRNA Was Followed by OSCAR mRNA—We demonstrated that OSCAR expression by TRANCE/M-CSF was significantly reduced in primary osteoclasts derived from MITF-/- mutant mice (Fig. 1). In addition, we showed that the combination of MITF and Pu.1 could synergistically activate the OSCAR promoter in transient transfection assays (Fig. 6). Therefore, to examine whether TRANCE-induced OSCAR gene expression is regulated by the level of MITF and/or Pu.1 mRNA during osteoclastogenesis, we analyzed the mRNA expression profiles of MITF, Pu.1, and OSCAR genes in TRANCE-treated RAW 264.7 or TRANCE/M-CSF-treated primary osteoclast cells. As shown in Fig. 8, MITF mRNA expression following treatment with TRANCE (RAW 264.7 cells) or TRANCE/M-CSF (primary osteoclasts) was induced early on day 1 and increased over time. In contrast to MITF, Pu.1 mRNA levels remained relatively constant at all time points. OSCAR mRNA was increased at later time points, namely at day 2 (Fig. 8A, RAW 264.7 cells) or day 3 (Fig. 8B, primary osteoclasts). These results suggest that TRANCE-induced MITF expression may be one of the regulatory factors for OSCAR gene expression during osteoclastogenesis. However, synergy with constitutively expressed Pu.1 might be required for dramatic up-regulation of OSCAR gene expression, which

Fig. 5. Transactivation of OSCAR promoter constructs by Pu.1. A, 0.1 μg of the pGL2 OSCAR 1.7 was transfected into 293T cells in the presence of a various amount of Pu.1-expressing plasmid as indicated. B, 0.1 μg of 1.7-kb mouse OSCAR promoter luciferase reporters containing wild type or mutations within the putative Pu.1 recognition sites were transfected into 293T cells with 1.0 μg of Pu.1-expressing plasmid. Results are representative of at least three independent sets of similar experiments (A and B). Data were normalized and presented as described in the legend to Fig. 3. C–E, sequence-specific recognition of Pu.1 binding sites in the OSCAR promoter by Pu.1 was examined by EMSA. GST-fused Pu.1 protein was incubated with 32P-labeled oligonucleotide probes in the absence or presence of cold competitors (unlabeled wild- or mutant-type probes) at 5-, 50-, and 250-fold molar excess concentrations. C, Pu.1 recognition site 1; D, Pu.1 recognition site 2; E, Pu.1 recognition site 3. Results are representative of at least two independent sets of similar experiments (C–E).
significantly exceeds up-regulation induced by either transcription factor alone.

**DISCUSSION**

Osteoclasts are bone-resorbing multinuclear giant cells derived from hematopoietic stem cells. Osteoclast development occurs in response to several osteotropic factors produced by osteoblasts/stromal cells, including 1,25-dihydroxyvitamin D₃, parathyroid hormone, and cytokines (1, 2). Soluble forms of M-CSF and TRANCE can support the osteoclastogenesis of mouse bone marrow or human monocyte cells (1–3). In addition, other factors produced by osteoblast/stromal cells can affect the differentiation and activation of osteoclasts. We have recently reported the identification of a novel member of the leukocyte receptor family, OSCAR, which has two Ig-like domains. OSCAR is specifically expressed in osteoclast cells and is involved in the differentiation of osteoclasts from mononuclear precursor cells (28).

Many transcription factors involved in bone development, including c-Fos, PU.1, and MITF, have been identified through studies of human patients and genetically modified mice (33). Mice lacking PU.1 are deficient in the macrophage/monocyte lineage and exhibit osteopetrosis (34). A mutant allele of the MITF gene, mi, encodes a protein lacking one arginine at amino acid position 217 of the MITF protein, which is critical for binding to target genes (18, 35). MITFmi/mi mutant mice show severe osteopetrosis caused by failure of mononuclear precursors to mature into multinuclear osteoclasts capable of bone resorption (19, 20). TRAP and cathepsin K are osteoclast-specific marker proteins expressed in functionally mature osteoclasts. Disruption of the genes encoding these proteins in mice leads to osteopetrosis (36, 37). Furthermore, MITF regulates expression of TRAP and cathepsin K genes during differentiation of osteoclasts (23, 27). Luchin et al. (27) has also reported that the DNA-binding domain of PU.1 binds to the HLH-LZ region of MITF and that the genetic and physical interactions between MITF and PU.1 are necessary for osteoclast gene expression and differentiation. In this study, we examined whether MITF and PU.1 also regulate osteoclast-specific OSCAR gene expression.

The promoter region of the OSCAR gene contains several recognition sequences for transcription factors including AP-1,
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PU.1 and MITF. Specifically, there are two potential E-box elements for MITF binding at −929 and −6 and three putative PU.1 recognition sites at −1472, −256, and −237 of the 1.7-kb mouse OSCAR promoter fragment (Fig. 2). OSCAR promoter activity is increased in a dose-dependent manner by wild type MITF and PU.1 (Figs. 3A and 5A). Using EMSA, we confirmed that MITF specifically binds to these two E-boxes and PU.1 binds to PU.1 recognition sites 2 and 3 but not PU.1 recognition site 1 (Figs. 4 and 5 (C–E)). Furthermore, the combination of wild type MITF and PU.1 synergistically activates OSCAR promoter activity (Fig. 6A). However, MITF-mi/mi was not able to synergize with PU.1 to activate OSCAR promoter activity (Fig. 6A). This result may explain the much lower OSCAR mRNA expression observed in MITFmi/mi osteoclasts compared with wild type osteoclasts (Fig. 1).

Using E-box or PU.1 recognition site mutants, we observed that the synergistic activation of the OSCAR promoter induced by the combination of MITF and PU.1 was not decreased in the E-box 1 mutant but was completely blocked in both E-box 2 and E-box 1, 2 double mutants (Fig. 6B). This result was unexpected, because binding of MITF to E-box 1 and E-box 2 sites was observed by EMSA (Fig. 4, A and B), and both E-box 1 and E-box 2 have the same nucleotide sequences (Fig. 2). These data suggest that sequences adjacent to MITF binding sites (E-boxes) are important for the interaction of MITF with PU.1. In addition, the synergistic activation of the OSCAR promoter was somewhat decreased in PU.1 recognition site 2 or 3 mutants but not in the PU.1 recognition site 1 mutant. The observation of synergistic activation in PU.1 recognition site 1 mutant is consistent with the inability of GST-fused PU.1 to bind to wild-type PU.1 recognition site 1 (Fig. 5C). Of note, the synergistic activation of the OSCAR promoter was also observed in the triple PU.1 recognition site mutant, which does not have obvious PU.1 transcription factor binding sites, although this activation was much lower compared with other mutants (Fig. 6, B and C). Luchin et al. has reported that independent binding sites for PU.1 and MITF are required for the synergistic transactivation of the TRAP promoter and that a ternary complex with DNA might be necessary for functional interaction to occur (27). In contrast, Yang et al. showed that PU.1 and the basic leucine zipper-containing transcription factor NF-IL6 synergistically activate the interleukin-1β core promoter in the absence of direct NF-IL6 binding to DNA recognition sites (38). According to this model, PU.1 appears to act as an anchor, tethering NF-IL6 to the interleukin-1β core promoter without NF-IL6 binding to its cognate site. In addition, Molkentin et al. (39) showed that the basic helix-loop-helix factor MyoD and its cofactor for regulation of muscle-specific genes, MEF2, require only a single binding site for either factor to induce synergistic transactivation of target genes. When considering that the MITF-mi/mi cannot bind its cognate recognition sequence but can physically bind to PU.1 (27), it is possible that functional binding of MITF to its DNA recognition site is necessary to elicit synergistic activation of the OSCAR gene in combination with PU.1. This suggestion is supported by the complete absence of synergistic activation of the OSCAR promoter in E-box 2 mutants. Furthermore, the observation that synergistic activation of the OSCAR promoter is detectable in the triple PU.1 recognition site mutant suggests that both of these mechanisms are involved in this synergistic transactivation of the OSCAR gene promoter by MITF and PU.1. It is also likely that the direct binding of PU.1 to its recognition sites 2 and 3 facilitates the interaction or tethering of PU.1 to MITF, which binds to E-box 2. However, more experiments are needed to test this speculation.

TRANCE receptor signaling through tumor necrosis factor receptor-associated factors activates multiple signaling pathways thought to be essential for osteoclast differentiation and functions. These include NF-κb, mitogen-activated protein kinase pathways, Src kinase, and phosphatidylinositol 3-kinase pathways (30, 31). Recently, Matsumoto et al. (40) reported that inhibition of the p38 signaling pathway in bone marrow-derived osteoclast precursor cells blocks the formation of multinuclear, functional osteoclasts that express TRAP in response to TRANCE. Furthermore, Mansky et al. (24) reported that MITF is a target of the MKK6/p38 signaling cascade initiated by TRANCE receptor signaling. Of note, OSCAR gene expression is also significantly increased by the MKK6/p38 signaling cascade. This result suggests that the modulation of MITF transcriptional activity by the MKK6/p38 signaling cascade is one of the important regulatory mechanisms for osteoclast-specific OSCAR gene expression.

Finally, the mRNA expression patterns of MITF, PU.1, and OSCAR gene in TRANCE-treated RAW 264.7 or TRANCE/M-CSF-treated cells (primary osteoclasts) reveal that MITF mRNA expression is induced much earlier than OSCAR gene expression. In contrast to MITF, PU.1 mRNA levels remain relatively constant at all time points. These results indicate that OSCAR gene expression is induced by up-regulation of MITF expression but not PU.1. Interestingly, although PU.1 is important for the synergistic transactivation of OSCAR, PU.1 expression is not limited to osteoclasts and can be observed in B lymphocytes, monocytes, granulocytes, megakaryocytes, mast cells, and immature erythroid cells (reviewed in Refs. 25 and 26). Therefore, it has been difficult to explain how PU.1 regulates osteoclast-specific expression of OSCAR. Our data suggest that TRANCE-induced up-regulation of MITF and MITF interaction with PU.1 leads to osteoclast-specific expression of OSCAR.

Taken together, our results strongly suggest that transactivation at both PU.1 and E-box sites mediates the synergistic enhancement of OSCAR gene expression. TRANCE-mediated OSCAR gene expression may involve both the up-regulation of MITF gene expression and increased MITF activity via the MKK6/p38/MITF signaling cascade.

Acknowledgments—We thank R. Dierova for help and support. We thank C. King and C. Perchonock for critically reading the manuscript.

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