Microphthalmia Transcription Factor Is a Target of the p38 MAPK Pathway in Response to Receptor Activator of NF-κB Ligand Signaling*

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Receptor activator of NF-κB ligand (RANKL) activates signaling pathways that regulate osteoclast differentiation, function, and survival. The microphthalmia transcription factor (MITF) is required for terminal differentiation of osteoclasts. To determine whether MITF could be a target of RANKL signaling, a phosphospecific MITF antibody directed against conserved residue Ser307, a potential mitogen-activated protein kinase (MAPK) site, was produced. Using this antibody, we could demonstrate that MITF was rapidly and persistently phosphorylated upon stimulation of primary osteoclasts with RANKL and that phosphorylation of Ser307 correlated with expression of the target gene tartrate-resistant acid phosphatase. MITF phosphorylation at Ser307 also correlated with persistent activation of the p38 MAPK, and p38 MAPK could utilize MITF Ser307 as a substrate in vitro. The phosphorylation of MITF and activation of target gene expression in osteoclasts were blocked by p38 MAPK inhibitor SB203580. In transient transfections, a constitutively active Rac1 or MKK6 gene could collaborate with MITF to activate the tartrate-resistant acid phosphatase gene promoter dependent on Ser307. Dominant negative p38 α and β could inhibit the collaboration between upstream signaling components and MITF in the transient assays. These results indicate that MITF is a target for the RANKL signaling pathway in osteoclasts and that phosphorylation of MITF leads to an increase in osteoclast-specific gene expression.

The osteoclast plays an important role in bone resorption in vertebrates during development and throughout life (1). This resorption is counterbalanced by new bone formation from osteoblasts. This process of coupling the actions of the bone-producing cells, the osteoblasts, and the bone-resorbing cells, the osteoclasts, is termed “bone remodeling,” a process necessary for maintaining a constant bone mass throughout the lifetime of vertebrate organisms. Disruption of this process in humans results in diseases of bone, including osteoporosis and hypercalcemia of malignancy (2, 3).

Osteoclasts differentiate from cells of the monocytic/macrophage lineage to become multinuclear, tartrate-resistant acid phosphatase (TRAP)1-positive cells capable of resorbing bone (4). Osteoclast differentiation is influenced by hormones and local factors produced by the osteoblasts and stromal cells (4). One local factor expressed by osteoblasts is receptor activator of NF-κB ligand (RANKL) (5–10). RANKL is a member of the tumor necrosis factor superfamily that is important for maturation of T- and B-cells as well as osteoclasts (7). Mice containing a targeted deletion in either the RANK receptor or RANKL lack differentiated osteoclasts and develop severe osteopetrosis (7–9).

RANKL/RANK signal through tumor necrosis factor receptor-associated factors to activate multiple signaling pathways thought to be important for osteoclast differentiation and function, including NF-κB, mitogen-activated protein kinase (MAPK) pathways, Src kinase, and phosphatidylinositol 3-kinase pathways (11, 12). Recently, it has been shown that inhibition of the p38 signaling pathway in bone marrow-derived osteoclast precursor cells by treatment with the drug SB203580 inhibited the formation of multinuclear, functional osteoclasts that expressed TRAP in response to RANKL treatment (13). In contrast, the drug PD98059, a specific inhibitor of the MAPK p42/44 pathway, had no effect on the differentiation of bone marrow cells. These results indicate that the p38 MAPK signaling pathway is involved in RANKL-induced differentiation of bone marrow-derived precursor cells (13).

The microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix leucine zipper protein (14, 15) closely related to the transcription factors TFE3, TFEB, and TFE2 (16–18). MITF regulates osteoclast target genes like TRAP, cathepsin K, and E-cadherin by binding to a 7-base pair conserved sequence TCANGTG found in the promoter regions of these genes (19–21). In situ hybridization experiments confirmed that MITF is expressed in osteoclasts beginning at the earliest stages of endochondral ossification of long bones (19). A mutant allele of the MITF gene, mi, encodes a protein product lacking one of four arginines in the basic region of the MITF protein critical for binding to target genes (14, 22). Osteoclast-like precursor cells derived from mice homozygous for the mi mutation are incapable of fusing to form multinuclear cells, lack a ruffled border, express low levels of TRAP and cathepsin K, and cannot efficiently resorb bone (19, 21, 23, 24). Based on the similarity of the phenotype of the bone marrow-derived precursor cells blocked for p38 MAPK activity and the mi/mi

1 The abbreviations used are: TRAP, tartrate-resistant acid phosphatase; RANK, receptor activator of NF-κB; RANKL, RANK ligand; MAPK, mitogen-activated protein kinase; MITF, microphthalmia transcription factor; CSF, colony-stimulating factor; HA, hemagglutinin; RIPA, radioimmune precipitation assay; Mops, 4-morpholinepropane-sulfonic acid; GST, glutathione S-transferase; OCL, osteoclast.

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osteoclasts-like cells, we hypothesized that MITF may be a target of the p38 MAPK in osteoclasts.

In this study, we investigate whether MITF is a potential target of the p38 MAPK in osteoclasts. We show that MITF was persistently phosphorylated on a conserved serine 307 in primary osteoclast-like cells in response to RANKL signaling and that phosphorylation of serine 307 correlated with expression of the target gene, TRAP. Further, p38 MAPK could phosphorylate serine 307 in vitro, and phosphorylation of MITF at serine 307 increased the ability of the factor to stimulate the TRAP promoter activity in transient transfection assays. We conclude that MITF may be a direct target of a RANKL/p38 signaling pathway that is necessary for osteoclast differentiation and function.

EXPERIMENTAL PROCEDURES

Culture and Analysis of OCLs—Hematopoietic precursors were obtained from the bone marrow of wild type mice. OCLs were grown in the presence of 50 ng/ml colony-stimulating factor-1 (CSF-1) for 3 days. CSF-1 was lowered to 5 ng/ml, and then RANKL was added at 100 ng/ml for the indicated times.

SB203580 (Calbiochem) was used at the indicated concentration, 10 μM, and was applied to the cells 30 min before stimulation with RANKL.

DNA Constructs and Transfections—For all experiments presented here, the melanocyte form of the MITF cDNA was used (14, 25). The MITF phosphorylation mutant, termed S307A/P308A, was made by replacing conserved serine and proline with alanine residues. The point mutant was verified by sequencing. The full-length form or point mutants were cloned into a vector providing the influenza hemagglutinin tag (HA) (Roche Molecular Biochemicals). Wild type TRAP promoter mutants were cloned into a vector providing the influenza hemagglutinin tag (HA) (Roche Molecular Biochemicals). Wild type TRAP promoter and the TRAP promoter containing the E box mutation were previously described (19).

Bacterial expression constructs of His-tagged MKK6(E) and p38α (26), dominant negative expression plasmids for p38α, β, γ, and δ isoforms (27), and MKK6(E) expression plasmid were all previously described (28).

DNA transfections of RAW 264 cells have been previously described (19).

Constitution of Cell Lines—RAW 264.7 cells were transfected with Superfect (Qiagen) following the manufacturer’s directions with either the wild type HA-tagged MITF or HA-tagged S73A/S307A/S307/ P308A/MITF, and cells were selected using 100 μg/ml Genetin (Invitrogen). Individual clones were picked and screened for expression of HA-MITF.

Preparation of Recombinant Proteins—Recombinant p38 isoforms and MKK6(E) were prepared by a previously described method (27). MITF was expressed in mammalian cells in the presence of 5-20 μM CSF-1 and purified to homogeneity by nickel-Sepharose affinity chromatography. The protein was cloned into pGEX2t (Pharmacia) and was purified to 95% purity by twofold affinity chromatography and eluted on an 8% SDS-PAGE. Phosphorylated proteins were visualized by autoradiography.

RESULTS

Production and Characterization of Antibodies Specific for MITF Phosphoserine 307—Osteoclasts that are grown in the presence of CSF-1 and RANK ligand but have been blocked for the activation of the p38 MAPK by the specific inhibitor SB203580 remain mononuclear and stain weakly for the osteoclast marker TRAP (15), a similar phenotype to the osteoclasts that are cultured from the mice homozygous for the mi mutation (23, 24). This led us to test the hypothesis that MITF might be a target of the p38 MAPK pathway during osteoclast differentiation. We analyzed the amino acid sequence of MITF and discovered a potential p38 phosphorylation site at serine 307 that is conserved among different species including human, mouse, and chicken (Fig. 1A). However, the serine at amino acid position 307 is not conserved among the other related transcription factors, TFE3, TFE3, and TFE2 (29).

To begin to investigate if MITF is phosphorylated at serine 307 in osteoclasts, an antibody that was specific for the phosphorylated serine at residue 307 was developed (see “Experimental Procedures”). For this purpose, the peptide PSTGLSpSPDVLN (corresponding to amino acids 301–312 of MITF) was synthesized and used to produce polyclonal rabbit serum. Following affinity purification, the specificity of the antibody for detecting phosphoserine 307 MITF in Western blotting experiments was tested with recombinant GST-MITF corresponding to amino acids 297–377. For these experiments, the GST-MITF region was incubated in vitro with activated p38 α and ATP.
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Fig. 1. Generation of phosphospecific MITF antibody. A, alignment of potential p38 MAPK phosphorylation sites in MITF. The sequence displayed is from amino acid 298 to 320. The conserved residues important for p38 MAPK phosphorylation are indicated in large boldface letters. B, phosphospecific MITF antibody recognizes only recombinant phosphorylated MITF. GST-MITF (amino acids 297–377) was incubated with nonradioactive ATP in the presence of the absence (lanes 1 and 3) or the presence (lanes 2 and 4) of His-tagged p38α. The top panel is a Western blot using phosphospecific MITF antibody. Lanes 1 and 2, wild type GST-MITF; lanes 3 and 4, S307A/P308A GST-MITF. Bottom panel, blot in the top panel reprobed with an antibody recognizing GST. C, phosphospecific MITF antibody recognizes HA-MITF stably expressed in RAW 264.7 cells upon stimulation with RANKL. Immunoprecipitates with HA antibody were run on an 8% SDS-PAGE. The top panel is a Western blot probed with the phosphospecific MITF antibody. The left half of the panel contains HA immunoprecipitates from cells that stably express HA-MITF containing a replacement of the serine 307 and proline 308 to alanines, whereas the right half of the panel contains HA immunoprecipitates from cells expressing HA-MITF with no change at serine 307 and proline 308. The bottom panel is the blot in the top panel reprobed with an antibody that recognizes the HA tag.

(Fig. 1B). These experiments showed that only GST-MITF that had been incubated with the p38 preparation was recognized by the antibody (Fig. 1B, lane 1 versus lane 2). Recombinant protein containing alanine substituted for serine at amino acid position 307 was not recognized by the phosphospecific antibody in either the presence or absence of activated p38 MAPK. (Fig. 1B, compare lanes 3 and 4 with lane 1). When the blot in Fig. 1B was stripped and reprobed with an antibody specific for the GST moiety, we were able to detect the recombinant protein in all four lanes (Fig. 1B, bottom panel).

To further characterize the phospho-Ser307-specific antibody, RAW 264.7 cell lines that stably expressed either HA-tagged MITF or S307A/P308A HA-tagged MITF were created. The cells were stimulated with RANKL for various times, and cell extracts were prepared and immunoprecipitated with the antibody recognizing the HA epitope. The phosphorylation status of MITF Ser307 was determined using the phosphospecific MITF antibody (Fig. 1C, top panel). This analysis demonstrated that Ser307 was phosphorylated following 15, 30, or 60 min with RANKL stimulation but that the antibody did not react with the S307A/P308A MITF protein. The bottom panel of Fig. 1C represents the blot in the top panel reprobed with an antibody that recognizes the HA tag to show approximately equal loading of the HA-containing immunoprecipitates.

MITF Is Rapidly and Persistently Phosphorylated in Osteoclasts after RANKL Stimulation—The anti-phospho-MITF antibody was used to determine the phosphorylation status of endogenous MITF in primary osteoclast precursors after stimulation by RANKL. Bone marrow-derived osteoclast precursors were obtained from wild type mice and cultured in the presence of CSF-1 for 3 days. At this time, RANKL was added to the bone marrow cell cultures, and nuclear extracts were prepared and analyzed by Western blotting (Fig. 2). Using the anti-phospho-Ser307 antibody, MITF phosphorylation could be detected following 30 min of RANKL stimulation and persisted following 24 h of continuous RANKL treatment (Fig. 2A, upper panel). When the same blot was reprobed with a nondiscriminating MITF antibody, approximately equal levels of MITF protein are seen in all lanes (Fig. 2A, lower panel). MITF appeared as a doublet with electrophoretic mobility of around 55 and 57 kDa in these experiments, and both bands are detected by the anti-phospho-Ser307 antibody (Fig. 2A). Previous work in melanocytes has also indicated that MITF is resolved as a doublet in extracts prepared from this cell type and that the upper band is due to c-Kit-mediated phosphorylation of conserved serine residue 73 (30). Ser73 is also phosphorylated in osteoclasts in response to CSF-1, accounting for the doublet band pattern in this cell line as well (31). As shown below (see Fig. 5) mutation of Ser73 to alanine results in a loss of the slower migrating band (see Fig. 5).

Real time PCR was used to measure expression of the MITF target gene TRAP (19) following RANKL stimulation of osteoclasts (Fig. 2B). These experiments demonstrated that TRAP expression was increased ~7-fold following 30 min of RANKL treatment and remained elevated 7-fold, following 24 h of...
p38 Can Phosphorylate MITF in Vitro—To provide additional evidence linking MITF phosphorylation to p38 MAPK, the effects of the specific p38 MAPK inhibitor, SB203580, on MITF Ser307 phosphorylation and TRAP gene expression were examined (Fig. 2, A and B). This analysis demonstrated that MITF Ser307 phosphorylation in response to RANKL could be inhibited by SB203580 pretreatment of osteoclasts (Fig. 2A, lane 3). Additionally, TRAP gene induction by RANKL could be reduced from 7- to 3-fold by SB203580 pretreatment (Fig. 2B).

p38 Can Phosphorylate MITF in Vitro—To provide additional evidence linking MITF phosphorylation to p38 MAPK, in vitro kinase reactions were performed. Two types of assays were used. First, p38 MAPK immunoprecipitated from RAW264.7 was used in immune kinase reactions with GST-MITF substrate (Fig. 3A). The immune kinase experiments demonstrated that the p38 immunoprecipitate prepared from RANKL-treated cells phosphorylated the GST-MITF protein, whereas one prepared from unstimulated cells did not (Fig. 3A, compare lane 1 and lane 3). Additionally, an MITF protein containing S307A/P308A mutations was a poor substrate in the assay, whether p38 was immunoprecipitated from RANKL-treated or -untreated cells (Fig. 3A, lanes 2 and 4).

In the second assay, the ability of recombinant p38α to phosphorylate GST-MITF was tested. Recombinant p38α, activated in vitro by constitutively active MMK6 (28), could phosphorylate wild type GST-MITF (Fig. 3B, lane 1). The level of phosphorylation of the Ser307 protein was ~5-fold higher than phosphorylation of S307A-mutated MITF protein (Fig. 3B, compare lanes 1 and 3). The phosphorylation of GST-MITF depended on the addition of both MMK6 and p38α (Fig. 3C, lanes 2 and 4), and GST alone was not phosphorylated by either kinase (Fig. 3C, lanes 5 and 6). In similar experiments, the p38 β isoform could also phosphorylate GST-MITF, but the p38 γ isoform could not (data not shown).

c-Jun N-terminal kinase MAP kinase could not phosphorylate GST-MITF substrates in vitro in either type of assay, under conditions where a GST-Jun substrate could be phosphorylated (data not shown).

Dominant Active Forms of Rac1 and MMK6 Can Collaborate with MITF to Superactivate the TRAP Promoter in Transient Assays—Rac1 and has been previously shown to be able to activate the p38 MAPK cascade (28). Further, MMK6 has been previously shown to be a MAPK kinase that is a specific activator of p38 MAPKs (28, 32). To provide additional evidence documenting MITF as a downstream target of the RANKL/p38 MAPK signaling pathway, we tested whether MITF’s ability to transactivate the TRAP promoter fused to a firefly luciferase reporter gene was stimulated by Rac1(15L) or MMK6(E), constitutive active forms of these molecules. Experiments were performed in the macrophage/osteoclast cell line RAW 264.7 (Fig. 4).

In these experiments, co-transfection of the TRAP reporter with MITF expression vector resulted in approximately a 10-fold increase in promoter activity compared with control basal activity in agreement with our previously published results (19) (Fig. 4). When either Rac1(15L) or MMK6(E) expression vectors were co-transfected with the TRAP reporter, there was an ~4-5-fold increase in TRAP promoter activity compared with basal activity (Fig. 4). When the combination of Rac1(15L) and MITF expression vectors was co-transfected with the TRAP promoter, there was a more than 100-fold increase in promoter activity relative to the control (Fig. 4, compare bar 1 with bar 5), an effect that was more than additive of the effects seen with MITF or Rac1(15L) expression vectors alone. When a TRAP reporter that contains point mutations in the E box-related MITF-binding site identified in our previous studies (19) was tested in combination with MITF and Rac1(15L), activation was abolished (Fig. 4, compare bar 5 with bar 6).

Co-transfection of MITF and MMK6(E) with either TRAP reporter produced similar results, a 100-fold activation of wild type reporter and no activation of the MITF-binding site mu-
transactivate the TRAP promoter to similar levels as observed in RAW264.7 cells (Fig. 5A) was studied in the transient transfection assays in RAW264.7 cells. Phosphorylation site in MITF collaboration with either Rac1(15L) or MKK6(E) alone or together. Activity is expressed as relative luciferase activity. The averages of three independent experiments performed in duplicate are shown, and the error bars indicate S.D. B, MITF proteins were expressed transiently in COS cells. Immunoprecipitation of 35S-labeled cell extracts with an HA-specific antibody is shown. Lane 1, HA vector; lane 2, HA-tagged wild type MITF; lane 3, HA-tagged MITF S307A/P308A; lane 4, HA-tagged MITF S73A/P74A (the Erk1 site) (30, 31). The immunoprecipitates were run on an 8% SDS-PAGE. The arrows indicate the positions of the wild type or mutated MITF protein. The specific MITF bands present with lower electrophoretic mobility probably represent the protein phosphorylated at serine 73 (see \"Results\" for details) (30, 31).

Serine 307 and Proline 308 Are Necessary for MITFCollaboration with Rac1 and MKK6—As discussed above, serine 307 is phosphorylated in vivo in response to RANKL signaling and is a substrate for p38 MAPK \textit{in vitro}. To test the role of this phosphorylation site in MITF collaboration with either Rac1(15L) or MKK6(E), the HA-MITF protein containing S307A/P308A (see Fig. 1) was studied in the transient transfection assays in RAW264.7 cells (Fig. 5A). When co-transfected with the TRAP reporter, the MITF S307A/P308A expression vector was able to transactivate the TRAP promoter to similar levels as observed with the wild type MITF gene (Fig. 5A). In contrast, MITF S307A/P308A co-transfected with constitutively active Rac1 or MKK6 could not activate the TRAP promoter to the same levels as wild type MITF (Fig. 5A). Mutation of other potential serine phosphorylation sites (e.g. an S73A point mutation in the previously characterized Erk phosphorylation site (30, 31)) had no effect on stimulation of MITF activity by either Rac1(15L) or MKK6(E) (data not shown).

To determine whether the alanine substitutions affected protein stability, we examined expression of HA-tagged MITF proteins following transfection of COS cells (Fig. 5B). In these experiments, both MITF wild type and S307A/P308A forms were again resolved as doublets. However, MITF with the S73A substitution ran as a single, faster migrating band, consistent with the previous finding that phosphorylation of this site by the Erk MAPK pathway results in the band with lower electrophoretic mobility (30, 31). No significant difference in the levels of the wild type MITF or MITF S307A/P308A proteins could be detected in this assay (Fig. 5B). Taken with the data above showing that S307A/P308A MITF can be stably expressed to the same extent as wild type protein in RAW264.7 cells (Fig. 1C, bottom panel), these results indicate that MITF protein stability is not grossly affected by these point mutations.

\textit{p38} \(\alpha\) and \(\beta\) Dominant Negative Can Inhibit Rac1/MITFActivation—There are four p38 isoforms (\(\alpha, \beta, \gamma, \text{ and } \delta\), encoded by separate genes (26, 33–35). We were interested in determining which isoform(s) of p38 was involved in osteoclastogenesis. Since SB203580 has been shown to inhibit only p38 \(\alpha\) and \(\beta\) isoforms at low doses used in the experiments presented above (36), it was likely that one or both of these isoforms were involved in osteoclast differentiation. Western analysis was performed with p38 antibodies specific for the p38 \(\alpha\) or \(\beta\) isoforms (26). The p38 \(\alpha\) isoform was detected in both RAW 264.7 and OCL whole cell extracts (Fig. 6A, lanes 1 and 2). However, we were unable to detect expression of p38 \(\beta\) in either RAW 264.7 or OCLs, although the antibody did recognize recombinant p38 \(\beta\) protein included on the blot as a positive control (Fig. 6A, compare lanes 5, 6, and 7, respectively).

To test if dominant negative versions of the four isoforms of p38 (27) could inhibit Rac1 activation of MITF, transient transfections were performed with RAW 264.7 cells (Fig. 6B). As shown above, co-transfection of MITF and Rac1 (15L) expression vectors with the TRAP reporter resulted in 100-fold activation of the TRAP promoter relative to the control (Fig. 6B, compare bar 1 with bar 4). When dominant negative expression vectors for p38 \(\gamma\) and \(\delta\) were co-transfected with MITF and Rac1 expression vectors, we detected no significant decrease in luciferase activity compared with when MITF and Rac1 expression vectors were co-transfected together (Fig. 6B, right panel). However, when expression vectors for the isoforms p38 \(\alpha\) and \(\beta\) were co-transfected with MITF and Rac1 expression vectors, the luciferase activity of the TRAP promoter was similar to levels seen when only MITF or Rac1 was co-transfected with the TRAP-luciferase reporter (Fig. 6B, left panel, compare bar 4 with either bar 5 or bar 6, respectively). Although our analysis indicated the p38 \(\beta\) isoform was not expressed in primary osteoclast-like cells, the fact that the dominant-negative version of the gene could still interfere with Rac1 activation of MITF is not surprising, given the high level of similarity between the p38 \(\alpha\) and \(\beta\) isoforms.

\textbf{DISCUSSION}

In this study, we present evidence indicating that MITF is a direct target of a RANKL/p38 MAPK signaling pathway and that activation of MITF by this pathway is necessary for efficient expression of TRAP. MITF is thus defined as a nuclear target for RANKL signaling that, along with p38 MAPK activity, is persistently, and not transiently, activated by RANKL. Studies on other signaling pathways activated by RANKL, specifically the IκB/NF-κB and c-Jun N-terminal kinase/c-Jun pathways, have not addressed the issue of persistence but have only studied activation within 30 min of RANKL treatment (10, 37, 38). RANKL can stimulate transient, acute inflammatory responses as well as promoting osteoclast differentiation (7). Thus, defining whether signaling pathways are activated in a transient or persistent manner is probably critical for understanding how RANKL promotes inflammatory responses as opposed to differentiation.

The p38 kinases constitute a distinct MAPK subfamily that plays a role in adaption, homeostasis, and stress responses (39). In addition, the p38 pathway has been implicated in the induction of myoblast differentiation (40, 41). There are interesting parallels between the role of p38 in muscle differentiation and in osteoclast differentiation. The myogenic MEF transcription factors are targets of p38 signaling (27, 42). Additionally, the p38 MAPK pathway is persistently activated and maintained during the whole process of myotube formation (41). As is the case for the p42/p44 MAPK kinase in neuronal

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**Fig. 5.** **Ser307** is necessary for collaboration with Rac1 or MKK6. **A**, activity of point mutants measured in transient transfection assays in RAW264.7 cells. TRAP luciferase reporter construct (2.5 μg) was co-transfected with 0.5 μg of expression vector for either wild type MITF, MITF containing the indicated point mutant, or 0.4 μg of Rac1 15L or MKK6(E) alone or together. Activity is expressed as relative luciferase activity (Fig. 1) was studied in the transient transfection assays in RAW264.7 cells. Phosphorylation site in MITF collaboration with either Rac1(15L) or MKK6(E) alone or together. Activity is expressed as relative luciferase activity. The averages of three independent experiments performed in duplicate are shown, and the error bars indicate S.D. **B**, MITF proteins were expressed transiently in COS cells. Immunoprecipitation of 35S-labeled cell extracts with an HA-specific antibody is shown. Lane 1, HA vector; lane 2, HA-tagged wild type MITF; lane 3, HA-tagged MITF S307A/P308A; lane 4, HA-tagged MITF S73A/P74A (the Erk1 site) (30, 31). The immunoprecipitates were run on an 8% SDS-PAGE. The arrows indicate the positions of the wild type or mutated MITF protein. The specific MITF bands present with lower electrophoretic mobility probably represent the protein phosphorylated at serine 73 (see “Results” for details) (30, 31).
cell differentiation (43), persistent activation of signaling appears necessary for the ability of the p38 MAPK pathway to promote cell differentiation in myoblasts and osteoclast precursors. In muscle differentiation, the identity of the factor that triggers p38 activation is unknown (41), unlike osteoclast differentiation, where RANKL is the factor that stimulates differentiation. The RANKL/p38 MAPK/MITF pathway provides a paradigm to define how persistent activation of signaling occurs.

MITF regulates distinct sets of target genes in different cell types, and a major question is what mechanisms allow for cell type-specific actions of MITF. The results presented here suggest that cell type-specific signaling events may contribute to the ability of MITF to selectively alter the expression of target genes in one cell type versus another. For example, the expression of RANK in osteoclast precursors distinguishes this cell type from melanocytes and thus allows for cell type-specific phosphorylation and activation of MITF. Importantly, the conserved Ser307 residue is not conserved among the related family members TFE3, TFEC, and TFEB (29). This is distinct from the conserved Ser31 residue, a target for the Raf/p42/p44 MAPK pathway (30, 31), a site conserved among the entire family (29). Thus, MITF may be regulated by the p42/p44 MAPK pathway in all cell types in which it is expressed, but phosphorylation of serine 307 may represent one mechanism of regulating MITF activity that is unique to osteoclasts.

How does phosphorylation at Ser307 modulate MITF activity in osteoclasts? Recent results suggest that two probable mechanisms should be considered. First, our laboratory has recently demonstrated that MITF and the Ets family member PU.1 interact uniquely in osteoclasts to regulate target genes and to promote osteoclast differentiation (44). One hypothesis is that phosphorylation of MITF by the p38 MAPK pathway increases the affinity of the interaction between MITF and PU.1 and thus increases the ability of this pair of transcription factors to stimulate osteoclast differentiation. A second hypothesis is that phosphorylation of MITF lowers its affinity for a co-repressor.

For example, in muscle differentiation, calcium/calmodulin signaling leads to a dissolution of a complex between MEF2 proteins and histone deacetylases and allows MEF2 to interact with the helix-loop-helix factor MyoD to trigger muscle differentiation (45). The two hypotheses are not mutually exclusive. It could be that phosphorylation of MITF on serine 307 leads to a lower affinity for a co-repressor and subsequent high affinity interaction with PU.1. Further analysis will be required to determine whether these hypotheses can account for the ability of p38 MAPK to activate MITF.

The work presented here indicates that osteoclast-specific signaling events may contribute to the ability of MITF to regulate osteoclast gene expression and differentiation. These results suggest a unique set of molecular targets that might allow modulation of osteoclast differentiation and activity in bone disorders caused by the uncoupling of osteoblast and osteoclast action (e.g. osteoporosis).

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