The receptor activator of NF-κB ligand (RANKL) induces various osteoclast-specific marker genes during osteoclast differentiation mediated by mitogen-activated protein (MAP) kinase cascades. However, the results of transcriptional programming of an osteoclast-specific cathepsin K gene are inconclusive. Here we report the regulatory mechanisms of RANKL-induced cathepsin K gene expression during osteoclastogenesis in a p38 MAP kinase-dependent manner. The reporter gene analysis with sequential 5′-deletion constructs of the cathepsin K gene promoter indicates that limited sets of the transcription factors such as NFATc1, PU.1, and microphthalmia transcription factor indeed enhance synergistically the gene expression when overexpressed in RAW264 cells. In addition, the activation of p38 MAP kinase is required for the maximum enhancement of the gene expression. RANKL-induced NFATc1 forms a complex with PU.1 in nuclei of osteoclasts following the nuclear accumulation of NFATc1 phosphorylated by the activated p38 MAP kinase. These results suggest that the RANKL-induced cathepsin K gene expression is cooperatively regulated by the combination of the transcription factors and p38 MAP kinase in a gradual manner.

Osteoclasts that play pivotal roles in bone morphogenesis, remodeling and resorption, differentiate from the hematopoietic myeloid precursors of macrophage/monocyte lineage (1–5). Osteoclast precursors expressing the receptor activator of NF-κB (RANK), a member of the tumor necrosis factor receptor family, differentiate into functional multinucleated osteoclasts in response to RANK ligand (RANKL) (6), also referred to as osteoclast differentiation factor (7), tumor necrosis factor-related activation-induced cytokine (8), or osteoprotegrin ligand (9). Mutant mice in which RANKL (10) or its receptor RANK (11) is disrupted exhibit severe osteopetrosis due to the lack of osteoclasts, indicating that the RANKL-RANK signaling system plays an essential role in osteoclast differentiation (12–14).

During osteoclastogenesis, stimulation of the RANK receptor triggers causal induction of osteoclast-specific marker genes defining the fate of the cell or its function. Among these genes, the expression of genes closely linked to bone resorption is specifically regulated by limited sets of transcriptional factors such as PU.1 (15, 16), c-Fos (17, 18), NF-κB (19, 20), or microphthalmia transcription factor (MITF) (21) expressed in osteoclasts, indicating that the RANKL-RANK signaling system plays an essential role in osteoclast differentiation (17). Recently, it has been revealed that the nuclear factor of activated T cells (NFAT) c1 transcription factor was induced by RANKL stimulation and then activated the TRAP gene promoter synergistically with c-Fos (24, 25). Thus, the inducibility of the osteoclast-specific genes is controlled by the combination of the RANKL-induced and noninducible transcription factors in a gradual manner.

A cysteine protease of the osteoclast-specific marker cathepsin K, which is capable of hydrolyzing extracellular bone matrix proteins, is highly expressed in osteoclasts (26). Pycnodysostosis is a human disease caused by congenital cathepsin K deficiency and indicates a crucial role of cathepsin K in the functional maturation of osteoclasts (27). The expression of the cathepsin K gene is controlled physiologically in vivo and in vitro by MITF (28). The mutant mi/mi mice that express a dominant negative form of MITF have the following characteristics, unpigmented small eyes, several intrinsic defects in melanocytes, mast cells, and multinucleated osteoclasts leading to osteopetrosis (21), which indicate essential roles of MITF in development of several cell type lineages. Cathepsin K
mRNA is still expressed in osteoclasts derived from the mutant mice (28, 29), suggesting a possible involvement of at least another modulator(s) upon the gene expression.

In osteoclast differentiation, the RANKL signaling is mainly mediated by recruiting adaptor molecules, TRAP proteins, that activate the mitogen-activated protein (MAP) kinase cascades (14, 30). Three distinct MAPs kinases, extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase, and p38 MAP kinase, identified in mammalian cells, are implicated as mediators to transmit nuclear signaling for cell growth and differentiation in response to stress and various cytokines (31, 32). Each of the kinases can activate specific transcription factors through direct phosphorylation prior to efficient expression of target genes. The p38 MAP kinase family members, consisting of p38α, p38β (33), p38γ (34), and p38δ (35) identified so far, serve as key determinants in cytokine-induced cell differentiation. In fact, we and others (23, 36) have reported that p38α MAP kinase activated by MAP kinase kinase 3 (MKK3) or MKK6, downstream of the RANKL signaling pathway, is critical for the relatively early phase of osteoclast differentiation. Recently, Mansky et al. (37) have reported that the p38α MAP kinase phosphorylates MITF, resulting in increasing of nuclear level of the TRAP gene. These findings suggest that the critical roles of p38 MAP kinase are to trigger and enhance the coordinated induction of the osteoclast-specific gene(s) through the modification of the limited sets of the transcription factor(s). However, the molecular mechanism of the cathepsin K gene expression remains to be fully defined in the context of RANKL-induced osteoclast differentiation mediated by the MAP kinase cascades.

This study shows that treatment with SB203580, a specific inhibitor of the p38 MAP kinase, markedly suppresses not only RANKL-induced cathepsin K gene expression in osteoclasts but osteoclast differentiation. After the analyses of the inducible mechanism of the RANKL-induced cathepsin K gene, we found here for the first time that NFATc1 interacts directly with p38 MAP kinase phosphorylated by p38 MAP kinase. These findings suggest that the transcriptional program of the RANKL-induced osteoclast-specific genes is controlled by limited sets of transcription factors at distinct stages of osteoclast differentiation. These studies give more detailed molecular analyses of the cathepsin K gene transcription leading the cells to undergo terminal differentiation of osteoclasts.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant soluble RANKL (sRANKL) was purchased from PeproTech (London, UK). Recombinant human macrophage colony-stimulating factor (M-CSF) was kindly provided by Morinaga Milk Industry Co., Ltd. (Tokyo, Japan). Polyclonal antibodies against p38 MAP kinase and phosphorylated p38 MAP kinase (Thr180/Tyr182) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Polyclonal antibody against NFATc1 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-NFATc1 monoclonal antibody was purchased from BD Biosciences. Alexa Fluor 546 goat anti-mouse and 488 goat anti-rabbit IgG antibodies were purchased from Molecular Probes, Inc. (Eugene, OR). Reverse transcription-PCR kit was obtained from Invitrogen. SB203580 and PD98059 were purchased from Calbiochem.

**Cell Culture**—Bone marrow cells were prepared by removing femurs and tibia from 6- to 8-week-old C57Bl/6J mice and flushing the bone marrow cavity with α-modified minimum Eagle’s medium (Sigma) containing 10% fetal bovine serum (FBS; Biological Industries, Israel) and 50 μg/ml penicillin/streptomycin (ICN Biomedicals, Inc. OH). After lysing the erythrocytes in lysing buffer (17 mM Tris, pH 7.65, 0.75% NH4Cl), cells were seeded at 1.5 × 10⁶ cells/well (0.5 ml) in 24-well plates in the presence of M-CSF. After a 3-day incubation, nonadherent cells were removed from the culture by pipetting and washed with phosphate-buffered saline (PBS). Adherent cells were further incubated in the presence of M-CSF and sRANKL for 5–6 days. The culture medium was replaced every 3 days with fresh complete medium. The cells were then washed and subjected to immunostaining, reverse transcription-PCR (RT-PCR), and immunoblot analysis.

The murine RAW264 cells (RIKEN, RCB0535) were maintained in α-modified minimal essential medium supplemented with 10% FBS (JRH Biosciences, Lenexa, KS) and 50 μg/ml penicillin/streptomycin as described previously. The HEK293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% FBS and 50 μg/ml penicillin/streptomycin.

Peripheral blood mononuclear cells (PBMC) were prepared from human heparinized blood by the Ficoll-Paque gradient centrifugation method and were incubated with human M-CSF. After 7 days, adherent macrophages were cultured with sRANKL for 3–5 days, and human osteoclasts were subjected to chlamydia immunoprecipitation (ChIP) assay.

**Detection of Cathepsin K mRNA Expression by RT-PCR**—Total RNA for cDNA synthesis was isolated from murine bone marrow cells and cultured cells as described previously (38). RNA was reverse-transcribed by using SuperscriptII reverse transcriptase, 1 mM dNTPs, 1 μg of oligo(dT) primers, and the supplied buffer (Invitrogen). RT-PCR reactions were carried out using the following primer pairs: 5′-AAATCTCTCCGGGT-3′ (sense) and 5′-TCATGGTTCCCAAGTG-GTTCC-3′ (antisense). PCR was carried out for 1 cycle at 95°C for 5 min, followed by 25 cycles at 95°C for 0.5 min, 57°C for 0.5 min, and 72°C for 0.5 min. The relative amounts of the cathepsin K mRNAs were normalized to that of the β-actin mRNA (39).

**Plasmid Construction**—The pcDNA3-NFATc1 plasmid was kindly provided by Dr. M. A. Brown (Emory University School of Medicine). The pcDNA3pu.1 plasmid was a kind gift of Dr. H. Sighn (Harvard Medical School). The MITF expression plasmid, pcDNAFLAG-MITF, was constructed as described previously (38). The expression plasmids for murine p38, pcDNA3p38, and the constitutive active form of MKK6 (MKK6CA), pSRaMKK6, were kindly provided by Dr. T. Sudo (RIKEN) and Dr. M. Karin (University of California, San Diego), respectively.

**Detection of Cathepsin K Gene Expression**—The full-length murine cathepsin K gene promoter plasmid, RAW264 cells were plated on 12-well plates at a density of 3 × 10⁴ cells/well the day before transfection. A total of 2 μg of plasmid DNA was mixed with Superfect (Qiagen; Santa Clarita, CA) and transfected into the cells following the manufacturer’s protocol. After 48 h of transfection, the cells were washed three times with PBS and then lysed in reporter lysis buffer (Promega; Madison, WI). Luciferase activity was then measured with a luciferase assay system (Promega) according to the manufacturer’s instructions. Luciferase activity was measured in triplicate, averaged, and then normalized to β-galactosidase activity to correct for transfection efficiency. β-Galactosidase activity was measured by using an α-nitrophenyl-β-D-galactopyranoside as a substrate.

**Electrophoretic Mobility-shift Analysis (EMSA)**—The full-length murine NFATc1 and Pu.1 cDNAs were transcribed by using T7 RNA polymerase, and the RNAs were then subjected to in vitro translation with rabbit reticulocyte lysate (Promega) as described previously (40). Oligonucleotide probes employed were labeled with [γ-32P]ATP using T4 polynucleotide kinase. DNA binding assays were performed by incubating 2 μl of reticulocyte lysate and 32P-labeled double-stranded oligonucleotides for 30 min at 30°C. For competition assays, a 200-fold excess of unlabeled double-stranded oligonucleotides was added in the reaction mixture. The samples were then resolved on a 4% polyacrylamide gel run in TBE buffer.

**Western Blot Analysis**—Immunoblot analyses and immunoprecipitations were performed as described. In brief, cells were lysed in lysis buffer (7.5, 150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Whole cell extracts (WCEs) were prepared by centrifugation at 10,000 × g for 15 min at 4°C after sonication of cells four times for 5 s. WCEs (30 μg) were electrophoresed on a 12% SDS-polyacrylamide gel and blotted onto a polyvinylidine difluoride membrane. Immunoblot detection was performed with the corresponding antibodies using an
p38 MAP Kinase Is Essential for the Induction of Cathepsin K Gene by RANKL—MAP kinases are important for transmitting various extracellular signals to the nucleus in diverse biological phenomena including cell differentiation (4, 42). We reported previously (38) that terminal osteoclast differentiation induced by RANKL is accompanied by the increased expression of cathepsin K mRNA. To determine whether the p38 MAP kinase signaling pathway is involved in the RANKL-induced cathepsin K gene expression, we analyzed cathepsin K mRNA by using semiquantitative RT-PCR in the presence or absence of specific kinase inhibitors. As shown in Fig. 1A, murine bone marrow macrophage (BMMs) cells were allowed to differentiate into mononuclear or multinucleated tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in the presence of RANKL and M-CSF for 3–5 days, and total RNAs were isolated for PCR analysis. Fig. 1B shows that RANKL treatment of BMMs cells increased the expression of cathepsin K mRNA by 19.8- and 28.9-fold at day 3 and 5, respectively, during osteoclast differentiation. When the cells were treated with RANKL in the presence of SB203580, a specific inhibitor of p38 MAP kinase (43–45), a dramatic reduction in the expression of cathepsin K mRNA as well as inhibition of osteoclast differentiation was observed. By contrast, PD98059, a specific inhibitor of MAP kinase for the MAPK/ERK pathway, had no effect on the RANKL-induced expression of cathepsin K mRNA. Similar results were obtained by using the murine monocyte/macrophage cell line, RAW264, which can differentiate into osteoclasts (data not shown). As control, the expression of β-actin mRNA was not affected by the treatment with RANKL in the presence or absence of the inhibitors. These results suggest that the induction of cathepsin K expression by RANKL is largely mediated by the p38 MAP kinase but not the MAPK/ERK signaling pathway.

Identification of RANKL-responsive Elements for NFAT Family and PU.1 in Cathepsin K Gene Promoter—Previous analyses of human cathepsin K gene promoter revealed several potentially important regulatory cis-elements, including the PU.1 and E box sites (46, 47). In addition to these regulatory elements, we also found two DNA sequences that closely matched the consensus sequence of the NFAT family (5'-GGAAAACTA-3'). These two cis-elements, which we term N1 and N2, are located from −580 to −572 (N1, 5'-GGAAAAACTA-3') and from −376 to −368 (N2, 5'-GGAAAAAGAT-3') in the upstream region from the transcription start site (Fig. 2). The E box site, which binds MITF and its family TFE3, was the only cis-element demonstrated previously to be important for the induction of the cathepsin K gene (28, 48). As a small amount of cathepsin K mRNA was still observed in osteoclasts from mi/mi mice (28), we sought to determine the importance of other potential cis-elements for its induction during terminal osteoclast differentiation.

To do this, we created chimeric reporter plasmids harboring...
the luciferase gene fused to various deletion mutants of the cathepsin K gene promoter region, and we then transfected these plasmids into RAW264 cells, which were subsequently induced to differentiate into osteoclasts by RANKL. As shown in Fig. 2, p-1108CathepsinLuc introduced into RAW264 cells displayed a 12-fold induction of the luciferase activity by RANKL. Deletion of the region from −1108 to −818, including the P1 and P2 sites and the most distal of the three E box sites, reduced the RANKL-induced luciferase activity by approximately 30%, consistent with the significance of the most distal E box site for cathepsin K gene expression as reported previously (28).

Somewhat surprisingly, however, the region downstream from −818 (p-818CathepsinLuc) retained the unexpectedly strong (−70%) of the p-1108CathepsinLuc inducibility of the luciferase activity by RANKL, raising the possibility that cis-elements other than the P1, P2, and E box sites also play additional roles in the RANKL inducibility of the cathepsin K gene. Indeed, the deletion of the region down to −516 (p-516CathepsinLuc) reduced the RANKL inducibility of the cathepsin K promoter by an additional 23%, and further deletion to −222 (p-222CathepsinLuc) virtually eliminated the inducibility. These results demonstrate that besides the E box sites in the maximum induction of the cathepsin K gene by RANKL.

Direct Binding of NFATc1 to the N1 and N2 Sites and of PU.1 to the P2 and P3 Sites—To determine whether the N1 and N2 sites as well as the P1, P2, and P3 sites directly bind NFATc1 and PU.1, respectively, we performed EMSAs by using the cathepsin K promoter probes containing each binding site for NFATc1 and PU.1. NFATc1 and PU.1, produced by in vitro translation using rabbit reticulocyte extract, were tested for binding to the labeled DNA probes. In these assays, NFATc1 was chosen among four members of the NFAT family, i.e., NFATp (NFAT1), NFATc1 (NFATc or NFAT2), NFAT3, and NFAT4, because NFATc1 is the only member of the NFAT family that is expressed during the terminal differentiation of osteoclasts (24, 25).

As shown in Fig. 3A, NFATc1 showed a strong binding to the N2 site (lane 2) and a moderate binding to the N1 site (lane 8). These bindings were specific for NFATc1, because the excess of the wild type competitor containing the mutated NFATc1 site (5'-TCTAAGAT-3') had much less effect. The presence of NFATc1 in the complexes for the N2 and N1 sites was confirmed further by the supershifts of these complexes with an anti-NFATc1 antibody (Fig. 3A, lanes 5 and 11). In similar sets of experiments, PU.1 was found to bind strongly to the P2 site and moderately to the P3 site; however, no binding was detected for the P1 site under the tested conditions (Fig. 3B).

Densitometric analysis of the observed complexes indicates that NFATc1 bound the N2 site 3.2-fold stronger than the N1 site and that PU.1 bound the P2 site 3.4-fold stronger than the P3 site, respectively. Thus, combined with the results from the deletion analysis of the cathepsin K promoter, these observations suggest that the P2, P3, N1, and N2 sites, but not the P1 site, play important roles for the expression of cathepsin K gene through the bindings of their cognate transcription factors.

Moreover, these results prompted us to determine whether these endogenous transcription factors, NFATc1 and PU.1, bound to the specific cathepsin K gene promoters in osteoclasts. To address the issue, the ChIP assay was carried out by using limited sets of primers that would specifically amplify a couple of promoter regions of the cathepsin K gene, containing either the NFATc1-binding N2 or the PU.1-binding P2 sites, as illustrated in Fig. 2. The results shown in Fig. 3C indicated that although all of the primer sets generated a PCR product of the predicted size from the chromatin DNA of PBMC, macrophages, and osteoclasts prior to the immunoprecipitation, the region including the NFATc1-binding N2 site was specifically immunoprecipitated with anti-NFATc1 antibody, and subsequently the amplified PCR products were detected significantly in osteoclasts, but not macrophages, by Southern blot analysis. As a negative control, no amplification by a primer set of the region from the exon 7 of human cathepsin K gene was detectable. The region containing the PU.1-binding P2 site immunoprecipitated with anti-PU.1 antibody was slightly amplified in PBMC and macrophages, yet a large amount of the fragment was markedly detected in RANKL-induced osteoclasts (Fig. 3D), suggesting that endogenous PU.1 is largely recruited to the P2 site in osteoclasts rather than in macrophages. In PBMC and macrophages, the fragment of the P2 site was enriched in immunoprecipitates of phosphorylated and acetylated histone H3, whereas recruitment of the modified histone H3 to the gene promoter was decreased in osteoclasts. Neither isotype-matched mouse nor rabbit IgG antibody precipitated both fragments containing the N2 and P2 sites, respectively. Thus, an extremely important implication of these results is that specific recruitment of these transcription factors to the specific pro-
moter in nucleosomes could regulate the transcriptional activation of the intrinsic cathepsin K gene during osteoclast differentiation.

Functional Interplay of NFATc1, PU.1, and MITF in the RANKL-induced Cathepsin K Gene Expression—To confirm further the functional roles of NFATc1 and PU.1 in cathepsin K gene regulation, we performed a reporter gene assay using a cell line of monocyte/macrophage lineage, RAW264 cells. As RAW 264 cells can differentiate into osteoclasts with RANKL stimulation, we utilized a constitutively active form of MKK6, MKK6CA, that activates p38 MAP kinase constitutively in place of RANKL to assess the inducibility of the cathepsin K promoter.

**Fig. 4.** Synergistic enhancement of promoter activity of cathepsin K gene by transcription factors together with activated p38 MAP kinase. A, RAW264 cells were co-transfected with 1.0 μg of p-1108CathepsinLuc plasmid and 0.3 μg of either NFATc1, PU.1, and/or constitutively active form of MKK6 (MKK6CA). After 48 h, luciferase activity was measured as described and normalized to the β-galactosidase activity of a co-transfected internal control plasmid. B, RAW264 cells were co-transfected with 0.3 μg of either NFATc1, PU.1, MKK6CA, and/or MITF. After 48 h, luciferase activity was measured as described and normalized to the β-galactosidase activity of a co-transfected internal control plasmid.

RANKL. Formaldehyde cross-linked chromatin from each cell was immunoprecipitated with control mouse IgG, anti-phosphorylated Ser10 (S10) and acetylated Lys14 histone H3 antibody, and anti-NFATc1 antibody and processed for PCR amplification as described under “Experimental Procedures” by using a primer set that would specifically amplify fragments containing the NFATc1-binding N2 site. As a positive control, PCR amplification was performed with input DNA from chromatin prior to the immunoprecipitation. Serving as a negative control, a primer set that would specifically amplify exon 7 of the cathepsin K gene was used. PCR products were resolved to agarose electrophoresis and then subjected to Southern blot analysis.
PU.1 increased the expression by 3.3- and 7.3-fold, respectively, whereas the simultaneous presence of both NFATc1 and PU.1 increased the expression by 9.6-fold. These results suggest that PU.1 has a stronger transcriptional activation than NFATc1 for the cathepsin K gene expression in the absence of activated p38 MAP kinase pathway, and that NFATc1 and PU.1 have an additive effect on the expression of the cathepsin K gene. We then tested the effect of the p38 MAP kinase pathway on cathepsin K gene expression by co-transfecting pSRoMKK6CA, which encodes an activated form of MKK6 that in turn activates p38 MAPK constitutively. As shown in Fig. 4A, in the presence of NFATc1 alone or NFATc1 and PU.1, MKK6CA increased the expression from 3.3- to 10.4-fold or from 9.6- to 20.7-fold, respectively. By contrast, in the presence of PU.1 alone, MKK6CA increased the expression only slightly from 7.3- to 11.7-fold, suggesting that the effect of MKK6CA occurs significantly through NFATc1. Taken together, these results demonstrate that NFATc1 and PU.1 cooperate for an additive effect on the expression of the cathepsin K gene and that the p38 MAP kinase pathway further augments the expression through NFATc1.

In terminal osteoclast differentiation induced by RANKL, the importance of MITF for the regulation of cathepsin K, TRAP, and osteoclast-associated receptor (OSCAR) is well established (22, 49). Given the dramatic effect of NFATc1 in the p38 MAPK-mediated (thus, RANKL-mediated) induction of the cathepsin K gene, we wished to determine the relative contribution of MITF, NFATc1, and MITF together with their functional cooperativity in the induction of the cathepsin K gene. As shown in Fig. 4B, MITF showed synergistic activation of the cathepsin K gene with PU.1 and NFATc1, which do not show synergy between themselves. MITF appeared to show a stronger synergy with PU.1 than with NFATc1. In the presence of all combinations of the two factors, namely MITF and NFATc1, MITF and PU.1, or PU.1 and NFATc1, a further increase in enzymatic activity was achieved when pSRoMKK6 was co-transfected, showing that MITF and NFATc1 can mediate the induction by p38 MAP kinase in any context of the assembled transcription factors. Furthermore, although the individual contributions of each factor and the kinase do not appear remarkable in themselves, the net effects of NFATc1, PU.1, MITF, and the activated MAPK pathway were dramatic and reached a 145-fold increase. These results suggest that although each transcription factor contributes to the expression of the cathepsin K gene to rather small degrees, the assembly of the transcription factors can induce a high level of cathepsin K expression via the MAP kinase pathway upon RANKL stimulation.

**Activated p38 MAP Kinase Induced by RANKL Phosphorylates NFATc1**—Next, we examined mRNA expression of the correlation between cathepsin K and the transcription factors, PU.1, MITF, and NFATc1 during osteoclast differentiation by semiquantitative RT-PCR analysis. Among these transcription factors, a large amount of NFATc1 mRNA was gradually expressed during osteoclastogenesis, which is consistent with the expression profile of cathepsin K gene induced by RANKL and M-CSF (Fig. 5B). On the other hand, it appeared that mRNA expression profiles of PU.1 and MITF were less affected. These results suggest that NFATc1 contributes to RANKL-induced cathepsin K gene expression as a key modulator.

The NFAT family is regulated via phosphorylation by mitogen-activated protein (MAP) kinases including ERKs, c-Jun N-terminal kinases, and p38 MAP kinases, which themselves are activated via phosphorylation by their cognate MKK3 or MKK6 (50, 51). In light of the our finding that NFATc1 mediates the p38 MAP kinase pathway for RANKL-induced cathepsin K gene expression, we tested if p38 MAP kinase and NFATc1 proteins were phosphorylated and synthesized, respectively, during osteoclast differentiation. As shown in Fig. 5A, macrophage cells isolated from the murine bone marrow were treated with M-CSF and RANKL to differentiate into bone marrow macrophages (BMMφ) and subsequently into multinucleated osteoclasts. In bone macrophage cells induced by M-CSF alone, neither increased expression nor phosphorylation of NFATc1 was observed (Fig. 5C and data not shown). Furthermore, only a small amount of phosphorylated p38 MAP kinase was observed at this stage. By contrast, in osteoclast cells induced by both M-CSF and RANKL, the amount of NFATc1 increased, consistent with previous findings that RANKL triggers the expression of NFATc1 in osteoclasts (24, 25). Moreover, phosphorylation of NFATc1 increased more dramatically compared with the increase in its amount (data not shown). Also, phosphorylation of p38 MAP kinase was markedly increased, whereas its protein level remained unchanged. These results suggest that in association with osteoclast differentiation induced by M-CSF and RANKL, the newly expressed NFATc1 undergoes phosphorylation by the activated p38 MAP kinase.

The NFAT proteins typically contain three highly conserved
serine-proline (SP) rich regions, designated as SP1 to SP3, that correspond to the regions from 198 to 214, from 235 to 244, and from 280 to 293 in NFATc1 (Fig. 6A) (51, 52). To test if SP regions in NFATc1 can indeed be phosphorylated by p38 MAP kinase in vitro, we prepared expression vectors for the GST fused serine-proline-rich region of NFATc1 (i.e. GST-SP1 to -SP3) (Fig. 6B). Activated p38 MAP kinase used for in vitro phosphorylation was obtained by immunoprecipitation with anti-pp38 MAP kinase antibody from HEK293 cells expressing pcDNA3p38 and pSRαMKK6CA by immunoprecipitation with anti-p38 MAP kinase antibody. Immunoprecipitates were subjected to in vitro immune complex kinase assay with the GST-SP1, -SP2, or -SP3 as substrate. Phosphorylated proteins (pNFATc1) were resolved by 12% SDS-PAGE and visualized by autoradiography. Closed arrowheads (pNFATc1) represent the phosphorylated NFATc1 (GST-SP2 and -SP3).

Moreover, to determine whether endogenous p38 MAP kinase interacts with NFATc1 in primary osteoclasts, we performed immunoprecipitation with anti-p38 antibody prior to immunoblot analysis by anti-NFATc1 antibody. As shown in Fig. 7C, the endogenous proteins significantly interacted with each other at day 3, but not at day 0, in RANKL-induced osteoclasts. In contrast, no interaction of them was detectable in the cells using rabbit normal IgG antibody as a control. In the same extracts from primary osteoclasts, treatment of RANKL indeed induced phosphorylation of p38 MAP kinase and de novo protein synthesis of NFATc1 (Fig. 7D). The endogenous interaction of NFATc1 with PU.1 was also observed in murine osteoclasts (data not shown). Taken together, these results suggest that NFATc1 associates endogenously with phosphorylated p38 MAP kinase and PU.1 during osteoclast differentiation.

Subcellular Localization of NFATc1 and PU.1 Transcription Factors—As p38 MAP kinase and NFATs shuttle between the cytoplasm and the nucleus depending upon their phosphorylation status (50, 52–54), we used immunocytochemical analyses to determine the subcellular localization of NFATc1 and p38 MAP kinase, as well as of PU.1, during the differentiation of murine bone marrow cells into osteoclasts. As shown in Fig. 8A, in murine bone marrow cells treated with M-CSF alone, p38

**Fig. 6.** p38 MAP kinase directly phosphorylates NFATc1. A, schematic diagram of the NFATc1 protein containing the structural domain of PIXITT, the three serine-proline-rich regions (SP1, -2, and -3), the two nuclear localization signals (NLS), and the nuclear export signals (NES). B, for in vitro kinase assay, GST fusion proteins of GST-SP1, -2, and -3 were prepared from Escherichia coli and resolved by 10% SDS-PAGE prior to Coomassie Blue (CBB) staining. C, activated p38 MAP kinase was purified from WCEs of HEK293 cells expressing pcDNA2p38 and pSRαMKK6CA by immunoprecipitation with anti-p38 MAP kinase antibody. Immunoprecipitates were subjected to in vitro immune complex kinase assay with the GST-SP1, -SP2, or -SP3 as substrate. Phosphorylated proteins (pNFATc1) were resolved by 12% SDS-PAGE and visualized by autoradiography. Closed arrowheads (pNFATc1) represent the phosphorylated NFATc1 (GST-SP2 and -SP3).
MAP kinase was present predominantly in the cytoplasm with an undetectable amount of NFATc1 in either the cytoplasm or nuclei. Slight cytoplasmic staining with anti-NFATc1 is probably nonspecific, because no NFATc1 mRNA expression was detected in BMMφ (Fig. 5, B and C) (24, 25). In the cells treated by both M-CSF and RANKL, however, a significant proportion of p38 MAP kinase re-localized from the cytoplasm into the nuclei, with concomitant expression of NFATc1, which also localized exclusively within the nuclei of the mononuclear and multinucleated osteoclasts. Moreover, there appeared to be a correlation between the expression levels of p38 MAP kinase and NFATc1, consistent with the induced transcription of NFATc1 by RANKL via p38 MAP kinase. By contrast, PU.1 was localized in the nuclei in a relatively constant amount, regardless of the differentiation status of the cells (Fig. 8B). Most interestingly, those cells that expressed higher levels of NFATc1 showed more fully differentiated morphology, namely multinucleated cells, regardless of the expression level of PU.1. Taken together, these results suggest that, upon the stimulation with RANKL followed by M-CSF, p38 MAP kinase and NFATc1 re-localize from cytoplasm into nucleus, whereas PU.1 remains in the nucleus at all stages. Given that NFAT proteins such as NFATp and NFAT4 typically re-localize from the nuclei to the cytoplasm upon phosphorylation by MAP kinases, the above-described observations that indicated the completely opposite behavior of NFATc1 were puzzling. To confirm further the nuclear re-localization of NFATc1 by p38 MAP kinase without the influence of PU.1 and other transcription factors involved in osteoclast differentiation, we introduced pcDNA3NFATc1, pcDNA3M2p38, and pSRoMK6 into HEK293 cells, and we determined the localization of NFATc1 in the presence of activated p38 MAP kinase. As shown in Fig. 8C, NFATc1 again localized predominantly in the cytoplasm in the absence of activated p38 MAP kinase, whereas in its presence, NFATc1 re-localized exclusively into the nuclei. Moreover, activation of p38 MAP kinase increased significantly the nuclear localization of NFATc1 by over 40% compared with that (data not shown) suggesting that p38 MAP kinase facilitates the re-localization of NFATc1 into the nucleus. These results confirm the nuclear localization of NFATc1 by p38 MAP kinase during osteoclast differentiation and suggest that the localization of NFATc1 is regulated distinctively from other members of the NFATc1 family.

**DISCUSSION**

**Regulation of the Cathepsin K Gene Transcription during Osteoclast Differentiation—Terminal differentiation of osteoclasts, as in the other differentiation systems, is presumed to involve multiple extracellular signals that are ultimately transmitted to the nucleus to induce the expression of specific genes critical for osteoclast differentiation. Our understanding of its molecular mechanisms, however, remains far from complete. In our continued effort to unravel the molecular basis of osteoclast differentiation, we used the induction of cathepsin K gene expression by RANKL as a model system, and we analyzed the roles of p38 MAP kinase and NFATc1 in the regulation of cathepsin K gene expression. In this study, we have revealed that RANKL allows p38 MAP kinase to phosphorylate NFATc1, which then relocates to the nucleus and binds to the cis-elements, N1 and N2, that are present in the upstream promoter region of the cathepsin K gene. NFATc1 appears to cooperate with both MITF and PU.1 on this promoter to attain the maximum level of transcription of the cathepsin K gene.**
Observations from our studies as well as others have enabled us to provide a schematic model of cathepsin K gene expression during osteoclast differentiation (Fig. 9). Osteoclast differentiation induced by M-CSF and RANKL proceeds through at least two stages distinguished by morphology as well as the expression level of osteoclast markers such as TRAP from osteoclast precursors to mononuclear osteoclasts, and finally to multinucleated osteoclasts as the cells undergo terminal differentiation. Osteoclast precursor cells, which are derived from myeloid lineage and are committed to differentiate into osteoclasts, can be induced by M-CSF to express RANK, the receptor for RANKL. The expression of RANK permits the cells to respond to subsequent RANKL stimulation and undergo morphological changes, resulting in the formation of multinucleated osteoclasts. Cathepsin K, initially expressed at a low level in osteoclast precursors, is induced by RANKL to a gradually higher level as the cells differentiate into multinucleated osteoclasts (Fig. 1) (55).

At the initial low level expression of cathepsin K observed in osteoclasts, PU.1 and MITF are likely to play major roles in maintaining its basal expression. Moreover, PU.1 and MITF are expressed at the earliest stage of osteoclast development and are essential for cells to commit to the osteoclast lineage, as mutant mice of PU.1 lack the committed precursors (15). Furthermore, M-CSF that initiates the first step of osteoclast differentiation is shown to activate MITF via MAP/ERK kinase (56) as well as to induce the RANKL receptor RANK. Subsequently, the expression of the cathepsin K gene will be greatly increased when RANKL together with M-CSF allows the precursors to differentiate into osteoclasts and then into multinucleated osteoclasts. The signal from RANKL is mediated by p38 MAP kinase that activates NFATc1 through phosphorylation of the SP1, SP2, and SP3 domains. The activated NFATc1 then relocates from the cytoplasm to the nucleus, where NFATc1 cooperates with MITF, and possibly AP-1 to further augment transcription of the cathepsin K gene, as is the case for TRAP (25, 49), because the AP-1 site is present in the promoter region of the cathepsin K gene. Thus, NFATc1, PU.1, MITF, and probably AP-1 form a complex on the cathepsin K promoter, which leads to the maximal level of cathepsin K gene transcription mediated by p38 MAP kinase. Indeed, RANKL-induced expression of other osteoclast-specific genes such as
Differentiation of Osteoclast Cells—

Members of the NFAT Family—

NFATc1 is a transcription factor whose localization is controlled by changes in the calcium ion concentration. It is a member of the NFAT family, which includes NFATc1, NFATc2, NFATc3, and NFATc4. These transcription factors are activated by calcineurin, a calcium/calmodulin-dependent protein phosphatase, and inhibited by calcineurin-activated phosphatase. NFATc1 is known to be expressed in osteoclasts, monocytes, and macrophages, and it is involved in the regulation of osteoclast differentiation and bone resorption.

Table 1

Summary of potential transcriptional regulation sites on osteoclast-specific genes

| Gene        | NFAT | PU.1 | MITF | AP-1 | NF-κB |
|-------------|------|------|------|------|-------|
| TRAP        | +    | +    | +    | +    | +     |
| Cathepsin K | +    | +    | +    | +    | +     |
| OSCAR       | +    | +    | +    | *    | +     |
| Integrin αv| +    | +    | +    | *    | +     |
| Integrin β3| +    | +    | +    | +    | +     |
| CalcitoninR | +    | +    | +    | +    | *     |
| Collagenase IV | + | + | ND  | + | +* |
| TCF1        | +    | +    | +    | +    | +     |

* Similarly matched motifs are indicated.
 ND indicates not detectable.

The transcription factors involved in the regulation of osteoclast differentiation are numerous, and their interactions are complex. In this context, the NFATc1 transcription factor plays a crucial role in the regulation of osteoclast differentiation. It is activated by RANKL (receptor activator of nuclear factor-κB ligand) in osteoclast precursors and stimulates the transcription of genes that are involved in osteoclast differentiation and function.

In summary, we provide evidence that NFATc1 and PU.1 together with MITF are functionally involved in the regulation of cathepsin K gene expression. These transcription factors cooperate functionally to regulate not only cathepsin K but other osteoclast-specific genes as well. The regulation of these genes is essential for the development of osteoclasts and bone resorption.

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