Although best known for its role in T lymphocyte activation, the calcineurin/nuclear factor of activated T cells (NFAT) signaling pathway is also known to be involved in a wide range of other biological responses in a variety of different cell types. Here we have investigated the role of the calcineurin/NFAT signaling pathway in the regulation of osteoclast differentiation. Osteoclasts are bone-resorbing multilaminated cells that are derived from the monocyte/macrophage cell lineage after stimulation with a member of the tumor necrosis factor family of ligands known as receptor activator of nuclear factor-κB ligand (RANKL). We now report that inhibition of calcineurin with either the immunosuppressant drugs cyclosporin A and FK506, or the retrovirally mediated ectopic expression of a specific calcineurin inhibitory peptide, all potently inhibit RANKL-induced differentiation of the RAW264.7 monocyte/macrophage cell line into mature multinucleated osteoclasts. In addition, we find that NFAT family members are expressed in RAW264.7 cells and that their expression is up-regulated in response to RANKL stimulation. Most importantly, we find that ectopic expression of a constitutively active, calcineurin-independent NFATc1 mutant in RAW264.7 cells is sufficient to induce osteoclasts to express an osteoclast-specific pattern of gene expression and differentiate into morphologically distinct, multinucleated osteoclasts capable of inducing the resorption of a physiological mineralized matrix substrate. Taken together, these data define calcineurin as an essential downstream effector of the RANKL-induced signal transduction pathway leading toward the induction of osteoclast differentiation and function, in particular, that the activation of the NFATc1 transcription factor is sufficient to initiate a genetic program that results in the specification of the mature functional osteoclast cell phenotype.

Bone is a dynamic tissue that is under a constant state of remodeling or homeostasis. This remodeling process is a delicate balance between the activities of osteoblasts, the cells that deposit bone, and osteoclasts, the cell type that is responsible for bone resorption (1, 2). Interference with this delicate balance can result in very serious human pathologies that affect bone integrity, such as osteoporosis and osteopetrosis. Accordingly, the molecular signaling pathways that regulate osteoblast and osteoclast differentiation and function have come under intense scrutiny.

Osteoclasts, the cells that resorb bone, are hematopoietically derived, multinucleated cells that arise from the monocyte/macrophage lineage (3). It is now clear that osteoclast differentiation is dependent upon the intimate cellular interaction of myeloid preosteoclast precursors with either osteoblasts or stromal cells and is influenced by a wide range of local factors (4, 5). In fact, a wealth of data has indicated that a member of the tumor necrosis factor family of ligands known as receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL; also known as ODF, OPGL, and TRANCE), which is expressed on both stromal cells and osteoblasts, plays an essential role in the regulation of osteoclast differentiation (6–8). Thus, a soluble recombinant form of RANKL (sRANKL) is sufficient to replace fully the requirement for osteoblast and stromal cell interactions in the induction of osteoclast differentiation in vitro cultures (6, 7), whereas agents that are known to stimulate osteoclastogenesis and bone resorption have been shown to increase the expression of RANKL on osteoblast cells (9–12). RANKL interacts with its specific receptor RANK, a member of the tumor necrosis factor receptor family, which is expressed on osteoclast precursors (8, 13). The importance of the RANKL/RANK receptor-ligand pair in the regulation of osteoclastogenesis is underscored by the observation that mice that are genetically deficient in either RANKL or RANK exhibit a severe osteopetrotic phenotype because of a profound defect in osteoclast differentiation (14–16). RANK is known to interact with a number of tumor necrosis factor receptor-associated factor (TRAF) signaling adaptor proteins, including TRAF-2, TRAF-5, and TRAF-6 (17), and stimulation of RANK activates a number of downstream signaling pathways, including mitogen-activated protein kinases, Src tyrosine kinase, phosphatidylinositol 3-kinase, and the transcription factors NF-κB and AP-1 (17–23). In fact, genetic studies in knock-out mice have revealed that the p50 and p52 subunits of NF-κB, as well as...
c-Fos, a component of the AP-1 transcription factor complex, are essential for efficient osteoclast maturation in vitro (24–26), whereas c-Src is required for mature osteoclasts to resorb bone (27). Furthermore, in vitro studies with specific chemical inhibitors have suggested important roles for the p38 mitogen-activated protein kinase and phosphatidylinositol 3-kinase in the regulation of RANKL-induced osteoclast differentiation (20, 22, 23). However, although RANKL/RANK signaling is clearly indispensable for osteoclast differentiation, our complete understanding of the contingent series of signaling events involved in RANKL-induced osteoclastogenesis is far from complete.

Previous studies have indicated that the immunosuppressant drugs cyclosporin A (CsA) and FK506 have pronounced effects on bone. Thus, the addition of either CsA or FK506 to in vitro bone organ or marrow cultures has been shown to inhibit both osteoclastogenesis and bone resorption induced by a variety of different agents including parathyroid hormone, calcitriol, prostaglandins, and cytokines (28–32). Furthermore, CsA has also been shown to inhibit bone resorption in isolated osteoclast cultures (33). Although these results suggest that CsA and FK506 are likely to inhibit an important step in the regulation of osteoclast differentiation and activation, this interpretation is complicated by the fact that these cultures contain both osteoclast precursors and RANKL-expressing osteoblasts and stromal cells. Hence, it is unclear from these latter studies whether the relevant cellular target for the inhibitory effects of these drugs is the osteoclast per se or alternatively, the osteoblast or stromal cell. This is an important issue to resolve because the inhibitory effects of CsA and FK506 on the generation of mature functional osteoclasts may help define a novel step in the regulation of osteoclastogenesis.

It is now well established that both CsA and FK506 are specific inhibitors of the calcium/calmodulin-regulated serine/threonine phosphatase, calcineurin (34). Calcineurin is best known for the role that it plays during T cell activation, where it acts to regulate the activity of the nuclear factor of activated T cells (NFAT) family of transcription factors and thereby couples stimulation of the T cell antigen-receptor to changes in the expression of cytokines and other important immunoregulatory genes (35, 36). In T cells, calcineurin is activated in response to the T cell receptor-induced increase in the intracellular calcium concentration. Once activated, calcineurin directly dephosphorylates NFAT proteins that are present in a hyperphosphorylated latent form in the cytoplasm and induces their rapid translocation into the nucleus, where in concert with nuclear partner proteins such as the AP-1 transcription factor complex, they are able to bind cooperatively to their target promoter elements and activate the transcription of specific NFAT target genes (35, 36). By potently inhibiting the in vivo activity of calcineurin, both CsA and FK506 are able to block the calcineurin-dependent nuclear translocation of NFAT proteins and thereby prevent the NFAT-dependent expression of a large number cytokines and other genes known to play an essential role in the initiation and regulation of the immune response. Although the calcineurin/NFAT signaling pathway is certainly best known for its role in the regulation of the immune response, it has become increasingly apparent that this pathway is also active in a wide range of cell types and tissues outside of the immune system (36, 37) and therefore plays a much broader role in the regulation of cell growth and development than previously appreciated.

In the current study we have investigated the potential role of the calcineurin/NFAT signaling pathway in the regulation of osteoclast differentiation. For a model system, we have used the RAW264.7 cell line because RANKL-induced stimulation of these monocyte/macrophage-like cells is known to be sufficient to induce osteoclast differentiation in the complete absence of either stromal cells, osteoblasts, or other exogenously supplied differentiation-inducing factors (8). We now report that the RANKL-induced differentiation of RAW264.7 cells is potently inhibited by both CsA and FK506. Furthermore, we find that ectopic expression of VIVIT-GFP, a previously characterized peptide inhibitor of calcineurin which is believed selectively to prevent calcineurin from activating NFAT proteins, but not other calcineurin-dependent signaling pathways (38), also potently inhibits RANKL-induced osteoclast differentiation. In fact, we show that members of the NFAT family of transcription factors are expressed in RAW264.7 cells and that their expression is up-regulated in response to stimulation with RANKL. Most importantly, we demonstrate that ectopic expression of a constitutively active NFATC1 mutant is sufficient to induce RAW264.7 cells to express an osteoclast-specific pattern of gene expression and undergo terminal differentiation into mature functional multinucleated osteoclast-like cells. Taken together, these data identify a novel role for the calcineurin/NFATC1 signaling pathway in the regulation of osteoclast differentiation and demonstrate that the NFATC1 transcription factor is sufficient to induce the transcriptional program leading to the acquisition of the mature osteoclast cell phenotype.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—RAW264.7 cells (American Type Culture Collection) were maintained at 37 °C, 5% CO2 in growth media: α-minimum Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum and 1% penicillin and streptomycin (Invitrogen). For osteoclastogenesis assays, cells were resuspended in fresh medium and cultured for 5–8 days in 96-well plates at cell density of either 500 or 3000 cells/well. Soluble RANKL (Peprotech) was added at 250 ng/ml as indicated, and CsA (Calbiochem) was used at 2 μg/ml, and FK506 (Calbiochem) at 1 ng/ml.

**Determination of Osteoclast Differentiation**—Osteoclast differentiation was assessed by measuring the cellular enzymatic activity of tartrate-resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts, and by the direct enumeration of TRAP-positive, morphologically distinct, multinucleated osteoclasts (39, 40). For the measurement of TRAP activity, cells were fixed in 10% (v/v) formalin for 10 min, washed in 95% (v/v) ethanol, and then incubated in 0.1 ml of phosphatase substrate (3.7 mM p-nitrophenyl phosphate in 50 mM citrate buffer, pH 4.6) in the presence of 10 mM sodium tetraborate at 25 °C for 30 min. After the incubation, the solution was from each well was transferred to a tube containing 0.1 ml of 0.1 M NaOH, and the absorbance at 410 nm was determined with a Dynatech MR5000 microplate reader. After the assay for TRAP activity, the cells were stained for TRAP with 0.1 mg/ml naphthol AS-MX phosphate and 0.6 mg/ml fast red violet LB salt in 0.1 M sodium acetate buffer, pH 5.0, containing 50 mM sodium tetraborate. All of the TRAP-positive cells with three or more nuclei were counted. Cultures were photographed with a Nikon Coolpix 4500 digital camera, and images were transferred with NikonView 5 software.

**Recombinant Retroviruses and Infection of RAW264.7 Cells**—The retroviral expression vectors pMSCV-GFP, M-SCV-VIVIT-GFP, and pMSCV-cNFATC1 have been described previously (41, 42). Recombinant retroviruses were produced by cotransfecting the pMSCV-GFP, pMSCV-VIVIT-GFP, or pMSCV-cNFATC1 proviral vector together with pSV5-G (Clontech), encoding the vesicular stomatitis virus glycoprotein, into the GP293 pantrophic packaging cell line (Clontech) using Lipofectamine Plus (Invitrogen). The medium was replaced after 24 h, and viral supernatants were harvested 2 days post-transfection and stored at -80 °C. For infections, 3,000 RAW264.7 cells were plated per well of a 96-well plate. The next day, the medium was replaced with 100 μl of viral supernatant containing 8 μg/ml Polybrene (Sigma), and plates were centrifuged at 2,000 rpm for 2 h at room temperature. After removal of viral supernatant, cells were cultured in growth medium for subsequent analysis.

**Determination of Functional Osteoclast Activity by Calcium Phosphate Resorption Assay and Pit Formation on Dentin Slices**—Cells were seeded onto 16-well BD BioCoat® Osteologic® calcium phosphate-coated quartz slides and incubated in α-minimum Eagle’s medium
supplemented with 15% fetal bovine serum, 1% penicillin and streptomycin, 50 μg/ml l-ascorbic acid, and 10 mM sodium β-glycerophosphate. The cells were infected with the appropriate recombinant retrovirus as described above, and the medium was changed after 24 h and three times weekly thereafter. After 10 days, bleach solution (6% NaOCl, 5.2% NaCl) was added to each well. The dish was agitated for 5 min and the medium/bleach mixture removed. Wells were washed twice in deionized water and then in running deionized water and allowed to air dry. Cultures were photographed as described above, and the transferred images quantified to determine the area of resorbed matrix using Scion Image® software. For direct examination of bone resorbing activity, cells were seeded in 24-well plates directly onto 0.5-mm thick dentin slices (a kind gift from Professor Hisashi Shinoda, Tohoku University Dental School, Sendai, Japan) and infected with either the MSCV-GFP or MSCV-cαNFATc1 retrovirus exactly as described above. After 10 days in culture, cells were removed by vigorous washing with bleach solution, dentin slices were washed in deionized water, and resorption pits were visualized by staining with hematoxylin and light microscopy. For visualization of resorption pits by scanning electron microscopy, dentin slices were first dehydrated in a series of ascending ethanol washes, then critically point dried under CO2. Dentin slices were then examined by scanning electron microscopy (Hitachi S3500) using a Robinson backscattered electron detector.

Semiquantitative Analysis of mRNA Expression by Reverse Transcription-PCR—Total RNA was isolated from cells using the RNeasy mini-prep method (Qiagen), and first strand cDNA was synthesized in 20-μl reactions using the Promega reverse transcription system following the manufacturer’s protocol. For analysis of the expression of NFAT family members, serial 2-fold dilutions of the first strand cDNA synthesis reaction were used for PCR amplification using Platinum Taq polymerase (Invitrogen) and the following gene-specific primer pairs: NFATc1 sense, 5′-CAACGCCCTTGACACCGATG-3′, and antisense, 5′-GGCTCTGACCTCTGATGCT-3′; NFATc2 sense, 5′-GGCCCATGTTGACGAGGA-3′, and antisense, 5′-GGGTTGGAGCTCTTCTGTCAG-3′; NFATc3 sense, 5′-CTTTCAGTTCTCTACCCCTTACCC-3′, and antisense, 5′-GCCAATACGTTCTCTTTTTGCC-3′; calcitonin receptor sense, 5′-AGGCTGAAGGAAAGCCCTGCA-3′, and antisense, 5′-GGAAGCTGTGATGCAATG-3′; integrin αv sense, 5′-GCCAGGATCAGCAGAGG-3′, and antisense, 5′-TGACCCCCGTATGAGCCT-3′; cathepsin K sense, 5′-GGGAGAAGTCCTCCAGAGG-3′, and antisense, 5′-GGCTCATTGAAGCCCTCAGCG-3′; calcitonin receptor sense, 5′-ACGGAGGAAAGCCCTGCA-3′, and antisense, 5′-GGCTCACAGGCTTTAAGT-3′. Thermal cycling conditions were: 22–27 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (TRAP, αv, and cathepsin K); 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min (NFATc3); 33 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min (calcitonin receptor). PCR products were resolved by 2% agarose gel electrophoresis and visualized by Vistra Green staining on a Storm PhosphorImager using ImageQuant software.

Statistics—Significance was determined by Scheffe’s F test.

RESULTS

CsA and FK506 Inhibit the RANKL-induced Osteoclast Differentiation of RAW264.7 cells—To determine initially the potential role of the calcineurin/NFAT signaling pathway in osteoclastogenesis, we first evaluated the effects of the specific calcineurin inhibitors CsA and FK506 on the ability of RANKL to induce the differentiation of RAW264.7 cells. Thus, cultures of RAW264.7 cells were stimulated with a range of sRANKL concentrations (3–100 ng/ml) in the presence and absence of either CsA or FK506, and osteoclast differentiation was assessed after 8 days by the production of TRAP enzymatic activity, a marker enzyme of osteoclast differentiation, and by the direct enumeration of multinucleated TRAP-positive cells. As indicated in Fig. 1, sRANKL stimulation induced a dose-dependent increase in both TRAP activity and the number of multinucleated TRAP-positive cells. Notably, both of these RANKL-induced parameters of osteoclast differentiation were significantly inhibited in the presence of either CsA or FK506 (Fig. 1). However, although the inhibitory effects of CsA and FK506 on sRANKL-induced TRAP activity were quite modest, both of these immunosuppressant drugs potently inhibited the appearance of morphologically distinct, TRAP-positive, multinucleated osteoclasts. Hence, these results indicate that although both CsA and FK506 inhibit the RANKL-induced differentiation of RAW264.7 cells, it is later steps in the
osteoclast differentiation pathway leading to fusion and multinucleation which appear to be more sensitive to the drugs, rather than the early production of TRAP activity.

**Ectopic Expression of the Specific Calcineurin Inhibitory Peptide VIVIT-GFP Blocks RANKL-stimulated Osteoclastogenesis**—The above results demonstrating that CsA and FK506 both inhibit the RANKL-induced osteoclast differentiation of RAW264.7 cells suggest that calcineurin activity is required for RANKL-induced osteoclastogenesis. To investigate this further, we took advantage of a 16-amino acid high affinity calcineurin-binding peptide identified by Rao and colleagues (known as VIVIT), which, when fused to the N terminus of GFP, has been shown to inhibit selectively the ability of calcineurin to activate NFAT proteins (38). To introduce this VIVIT-GFP fusion protein into RAW264.7 cells efficiently, we utilized our previously described retroviral vector in which VIVIT-GFP is under the transcriptional control of the MSCV long terminal repeat (42). By pseudotyping this recombinant retrovirus with the vesicular stomatitis virus glycoprotein G we were able to generate high titer virus capable of routinely infecting ≥95% of RAW264.7 cells (Fig. 2A). This high level of infection efficiency obviates the laborious process of isolating and expanding clonal cell lines and means that we are able to analyze bulk populations of cells immediately after infection. To determine the effects of VIVIT-GFP on RANKL-induced osteoclast differentiation, RAW264.7 cells were infected with either MSCV-VIVIT-GFP or a control MSCV-GFP retrovirus and then induced to differentiate in the presence of sRANKL. As indicated in Fig. 2B, ectopic expression of VIVIT-GFP in RAW264.7 cells modestly inhibited the RANKL-induced increase in TRAP activity. However, like our results with CsA and FK506, we found that ectopic expression of VIVIT-GFP potently blocked the RANKL-induced formation of morphologically distinct, TRAP-positive, multinucleated cells (Fig. 2, C and D). To determine whether calcineurin was also required for the RANKL-dependent induction of osteoclast functional activity, we tested the effects of VIVIT-GFP on the ability of RANKL to induce the resorption of a synthetic bone substrate. For this analysis we took advantage of BioCoat® Osteologics® slides that are coated with calcium phosphate and which have been extensively used in the analysis of osteoclast function (43–45). Thus, RAW264.7 cells infected with either the control MSCV-GFP or the MSCV-VIVIT-GFP retrovirus were plated on the BioCoat® Osteologics® slides, then treated with sRANKL, and the extent of calcium phosphate resorption was determined after 10 days in culture. As shown in Fig. 2E, we found that RAW264.7 cells infected with the control retrovirus readily resorbed the calcium phosphate matrix when stimulated with RANKL, whereas the RANKL-induced resorption activity of cells infected with MSCV-VIVIT-GFP was markedly reduced. Taken together with the effects of CsA and FK506, these data strongly suggest that calcineurin activity is required for the RANKL-stimulated differentiation of RAW264.7 cells into morphologically distinct, TRAP-positive, multinucleated functional osteoclasts. Moreover, because VIVIT-GFP has been demonstrated to block preferentially the ability of calcineurin to dephosphorylate NFAT proteins, but not other calcineurin substrates, these data raise the possibility that the NFAT family of transcription factors might play a role in the regulation of RANKL-induced osteoclast differentiation and function.

**NFAT Family Members Are Expressed in RAW264.7 Cells, and Their Expression Is Induced by Stimulation with RANKL**—Although NFAT proteins were originally identified in cells of the immune system, it has become increasingly apparent that these proteins are also expressed in a wide variety of other cell types. To determine whether NFAT family members are expressed in RAW264.7 cells we performed reverse transcription-PCR analysis on mRNA isolated from either unstimulated or RANKL-induced RAW264.7 cells using oligonucleotide primers specific for individual members of the NFAT family. As shown in Fig. 3, we find that NFATc1 and NFATc3 mRNAs are indeed expressed at a low level in resting nonstimulated RAW264.7 cells and that that this level of expression is increased further in response to stimulation with sRANKL. The mRNA for NFATc2 was barely detectable in resting RAW264.7 cells, but like NFATc1 and NFATc3, its expression was also induced after stimulation with sRANKL. NFATc4 mRNA was not detected in RAW264.7 cells (data not shown).

**Ectopic Expression of a Constitutively Active NFATc1 Mutant in RAW264.7 Cells Is Sufficient to Induce Osteoclastogenesis**—Having established that NFAT family members are expressed in RAW264.7 cells and that the RANKL-induced differentiation of RAW264.7 cells into multinucleated TRAP-positive bone-resorbing osteoclasts is potently inhibited by VIVIT-GFP, which is believed to inhibit selectively the ability of calcineurin to activate NFAT proteins (38), we next wished to assess directly the potential contribution of the NFAT proteins themselves to osteoclast differentiation. Toward this end we took advantage of our previously characterized constitutively active NFATc1 mutant (caNFATc1), which is known to be constitutively localized to the nucleus, bind DNA with high affinity, and is capable of activating endogenous chromatin-embedded NFAT target genes (41). We used this NFATc1 mutant to determine whether activation of the NFATc1 signaling pathway by itself in RAW264.7 cells was sufficient to induce osteoclast differentiation. Thus, RAW264.7 cells were infected with either a control MSCV-GFP retrovirus or our previously described MSCV-caNFATc1 virus, and the effects on osteoclast differentiation were examined. As shown in Fig. 4, enforced expression of caNFATc1 in RAW264.7 cells was sufficient to induce TRAP activity and the appearance of large morphologically distinct, TRAP-positive, multinucleated cells in the complete absence of RANKL signaling. Conversely, in the absence of RANKL stimulation, cells infected with the control virus never underwent a morphological change toward the osteoclast phenotype. Notably, expression of caNFATc1 significantly increased the kinetics of osteoclast differentiation, as multinucleated cells appeared as early as 48 h after infection with the MSCV-caNFATc1 virus compared with the 4–6 days required for the initial appearance of these cells after RANKL stimulation (data not shown).

To determine whether these caNFATc1-induced TRAP-positive multinucleated cells were capable of mediating mature osteoclast function, we examined their activity in two independent functional osteoclast assays. First, we assessed the ability of either control or caNFATc1-expressing RAW264.7 cells to resorb a calcium phosphate substrate. Thus, RAW264.7 cells infected with either the control MSCV-GFP or MSCV-caNFATc1 retrovirus were plated on BioCoat® Osteologics® slides, and the extent of calcium phosphate resorption was determined after 10 days in culture. As shown in Fig. 4D, we found that RAW264.7 cells infected with the caNFATc1 virus, but not the control virus, were capable of mediating significant resorption of the calcium phosphate matrix. Second, we examined whether caNFATc1-expressing RAW264.7 cells were capable of directly resorbing a physiological mineralized substrate, dentin. As shown in Fig. 4E, after 10 days in culture on dentin slices, cells infected with the caNFATc1 virus formed clearly discernible resorption pits as visualized by hematoxylin staining, whereas no such pits were observed on dentin slices cultured with control MSCV-GFP virus-infected RAW264.7 cells.
under identical conditions. To establish formally that these latter hematoxylin staining areas detected on the dentin slices cultured with caNFATc1-expressing RAW264.7 cells truly represented the formation of caNFATc1-induced resorption pits and were not merely an artifact caused perhaps by the staining of residual cell debris, we performed scanning electron microscopy of the dentin slices. As clearly indicated in Fig. 4F, this analysis revealed that the hematoxylin staining areas detected do indeed correspond to resorption pits formed on the surface of the dentin slice. Taken together as a whole, these data provide clear evidence that the ectopic expression of caNFATc1 is sufficient to induce RAW264.7 cells to acquire mature osteoclast functional activity.

Finally, to gain independent molecular evidence that activation of the NFATc1 transcription factor is capable of inducing RAW264.7 cells to undergo terminal osteoclast differentiation,
we determined the effects of caNFATc1 on the expression of a panel of genes that are known to be expressed selectively in terminally differentiated osteoclasts. As shown in Fig. 5, expression of caNFATc1 in RAW264.7 cells is sufficient to induce the expression of TRAP, the αv and β3 integrin subunits, cathepsin K, and the calcitonin receptor, which are all known to be induced during RANKL-stimulated osteoclastogenesis (1, 46–49). This result indicates that activation of the NFATc1 transcription factor is able to mimic RANKL-induced signals and induce RAW264.7 cells to acquire molecular markers associated with terminally differentiated osteoclasts. Taken together, therefore, the collective data presented in Figs. 4 and 5 provide strong and compelling evidence that the activation of the NFATc1 transcription factor is, in and of itself, sufficient to induce the differentiation of RAW264.7 cells into cells with a morphologically distinct, terminally differentiated functional osteoclast phenotype.

**DISCUSSION**

The calcineurin/NFAT signaling pathway was originally identified in T cells, where it is known to play a key role in the regulation of immunologically important cytokine genes during the initiation of the T cell immune response (35, 36). However, it has now become increasingly clear that this signal transduction pathway is also active in a number of other distinct cell and tissue types and plays a much broader role in the regulation of cell growth and development than was initially appreciated. These additional roles include the regulation of cardiac valve morphogenesis, patterning of the vasculature, cardiac hypertrophy, angiogenesis, regulation of chondrocyte differentiation, and multiple roles in the regulation of skeletal muscle, such as myogenesis, muscle fiber-type gene expression, and myocyte hypertrophy (36, 37, 50–54). Our current data, along with other recent results (55, 56), now extend to osteoclasts, the list of cell types that take advantage of the calcineurin/NFAT signaling pathway as a means of regulating cell fate choices and cellular functions.

In our initial studies we used specific inhibitors of the calcium/calmodulin-regulated serine/threonine phosphatase, calcineurin, together with the well established RAW264.7 monocyte/macrophage-like/preosteoclast cell line to investigate the potential role of calcineurin in the regulation of RANKL-induced osteoclastogenesis. As a result of these studies, we found that the structurally distinct immunosuppressant drugs CsA and FK506, which are each known to inhibit the calcineurin calcium-induced enzymatic activity specifically (34), both potently blocked the RANKL-induced differentiation of RAW264.7 cells into mature TRAP-positive, multinucleated osteoclasts. Similar inhibitory effects of CsA and FK506 on the RANKL-induced osteoclastogenesis of bone marrow-derived monocyte/macrophage precursors have also been reported recently (55, 56). In addition to the effects of the immunosuppressant drugs, we found that ectopic expression of VIVIT-GFP, a previously characterized specific peptide inhibitor of calcineurin (38), also potently inhibited the ability of RAW264.7 cells to undergo terminal osteoclast differentiation in response to treatment with RANKL. Taken together, the observation that three structurally distinct inhibitors of calcineurin all act independently to block RANKL-induced osteoclast differentiation provides strong and compelling evidence to implicate calcineurin as an essential downstream effector of the RANKL-induced signal transduction pathway leading toward the induction of terminal osteoclast differentiation and thereby helps define a novel role for calcineurin in the regulation of osteoclastogenesis.

Interestingly, the VIVIT-GFP calcineurin inhibitory peptide used in our studies was initially reported by Rao and colleagues (38) to inhibit selectively the ability of calcineurin to activate NFAT proteins, but not other downstream targets such as NF-κB. Hence, our observation that VIVIT-GFP potently blocked RANKL-induced osteoclastogenesis suggested a potential role for NFAT family members in the regulation of RANKL-induced osteoclast differentiation. This was an initially surprising notion because NFAT family members were not known previously to be expressed in cells of the monocyte/macrophage lineage. However, as revealed by our reverse transcription-PCR analysis, NFAT family members NFATc1, NFATc2, and NFATc3 are indeed expressed in RAW264.7 cells. Moreover, we found that their expression is up-regulated in response to stimulation of the RANKL/RANK signaling pathway, thereby establishing a direct link between a signal known to be pivotal in the induction of osteoclast differentiation and the increased expression of members of the NFAT family of transcription factors. Similar RANKL-induced increases in the expression of NFATc1 mRNA in bone marrow-derived monocyte/macrophage precursors have also been reported recently (55, 56). In fact, these later studies also revealed that RANKL stimulation leads not only to an increase in NFATc1 gene expression, but also to the translocation of NFATc1 proteins from the cytoplasm into the nucleus.

A significant question that is raised by these studies is how does RANKL-induced stimulation of RANK lead to the activation of the calcium/calmodulin-dependent phosphatase, calcineurin, and in turn the NFAT family of transcription factors? In this regard, although RANK-mediated signal transduction has been studied extensively for a number of years, much of the focus has been on the mechanisms by which RANK stimulation couples to the activation of the mitogen-activated protein kinase, AP-1, and NF-κB signaling pathways (57). In fact, it is only very recently that RANKL treatment has also been shown to lead directly to an increase in the intracellular calcium concentration (55, 56). However, potential insights into the molecular mechanism underlying RANKL-induced changes in intracellular calcium are provided by the analysis of RANKL/RANK signaling in the regulation of angiogenesis in endothelial cells, which has revealed that RANKL stimulation can activate calcium-dependent signaling events via the effects of the Src tyrosine kinase on phospholipase C (58). The Src tyrosine kinase is coupled to RANK via its interaction with the cytoplasmic adaptor protein, TRAF-6, which is known to play an essential role in the regulation of RANKL-induced osteoclast differentiation and the stimulation of bone resorbing activity (59). In fact, in addition to playing a role in the regulation of...
RANK-induced phospholipase C activity, the Src tyrosine kinase has also been shown to play a role in the RANK-induced regulation of protein kinase B (18). This is of interest because protein kinase B is known to phosphorylate and inhibit the activity of glycogen synthase kinase-3 (60), which is known to be a potent inhibitor of NFATc1 activity (61, 62). Accordingly, we believe that the RANK-induced activation of Src is likely to affect the activity of the calcineurin/NFATc1 signaling pathway in two distinct ways. First, the TRAF-6/Src-dependent activation of phospholipase C and the subsequent production of the calcium mobilizing agent inositol trisphosphate lead to an increase in the intracellular calcium concentration which in turn presumably activates calcineurin, thereby inducing the dephosphorylation of NFATc1 and its rapid transport into the nucleus. Second, the TRAF-6/Src-dependent activation of protein kinase B-dependent likely results in the phosphorylation and subsequent inhibition of the principal NFATc1 inhibitory kinase, glycogen synthase kinase-3, thereby preventing this kinase from directly phosphorylating active NFATc1, which is known both to inhibit its DNA binding activity and promote its export back into the cytoplasm (61, 62). In fact, support for this proposed role of Src in the regulation of the calcineurin/NFAT signaling pathway is provided by the observation that activation of Src family members has previously been shown to be sufficient to induce fully the calcineurin-dependent activation of NFATc1 in osteoclasts.

**Fig. 4.** Ectopic expression of a caNFATc1 mutant is sufficient to induce RAW264.7 cells to acquire a mature functional osteoclast-like phenotype. RAW264.7 cells infected with either control MSCV-GFP or MSCV-caNFATc1 virus encoding a constitutively active form of NFATc1 (caNFATc1) were left unstimulated and analyzed for parameters of osteoclast differentiation after 8 days in culture. A, effect of ectopic expression of caNFATc1 on TRAP activity. B, effect of ectopic expression of caNFATc1 on the number of multinucleated (MNC) TRAP-positive cells. ND, none detected. C, morphological appearance of cultures infected with either the control MSCV-GFP retrovirus (left panel) or the MSCV-caNFATc1 retrovirus (right panel). D, effect of ectopic expression of caNFATc1 on the ability of RAW264.7 cells to resorb a calcium phosphate substrate. RAW264.7 cells grown on Bio-Coat® Osteologic® calcium phosphate-coated quartz slides were infected with either the control MSCV-GFP (left panel) or the MSCV-caNFATc1 virus (middle panel), and representative images showing the appearance of the matrix after 10 days of culture are presented. Quanti- tation of the area of resorption induced by either control or MSCV- caNFATc1 virally infected cells was determined as described under “Experimental Procedures” and is presented as mean area of resorption (in mm²) (right panel). Results are the means ± S.E. of responses from six cultures. * p < 0.05 versus cells infected with control retrovirus. E, effect of ectopic caNFATc1 expression on the ability of RAW264.7 cells to form resorption pits on dentin slices. RAW264.7 cells infected with either control MSCV-GFP (left panel) or the MSCV-caNFATc1 (middle panel) retrovirus were incubated on dentin slices for 10 days, at which point cells were removed, and resorption pits were visualized by hematoxylin staining. Resorption pits appear as dark spots stained with hematoxylin. Quantitation of the data showing mean area of resorption (in mm²) ± S.E. is presented (right panel). ND, none detected. F, representative scanning electron microscope images illustrating the formation of resorption pits in the surface of dentin slices cultured in the presence of caNFATc1-expressing cells. Original magnification, ×150 (left) and ×100 (right), respectively.

**Fig. 5.** Effect of constitutively active NFATc1 on gene expression in RAW264.7 cells. RAW264.7 cells infected with either the control MSCV-GFP or the MSCV-caNFATc1 virus were cultured for 6 days, at which point RNA was isolated for reverse transcription-PCR analysis using gene-specific primers for TRAP, alpha V, beta 3, cathepsin K, calcitonin receptor, and cyclophilin. PCR products were resolved by 2% agarose-gel electrophoresis and visualized by Vistra Green staining using a Storm PhosphorImager.
genes including TRAP, cathepsin K, the calcitonin receptor, to these morphological and functional criteria, we also observe an oclast-like bone-resorbing phenotype. Importantly, in addition to these morphologically distinct, TRAP-positive, multinucleated osteoclast-like bone-resorbing cells, we also observe that the activation of NFATc1 transcription factor is sufficient to induce the program of transcriptional events leading to the acquisition of the mature osteoclast phenotype. The importance of the NFATc1 transcription factor to the regulation of osteoclastogenesis is underscored further by two other recent sets of studies. First, Ishida et al. (55) found that the inhibition of NFATc1 expression in RAW264.7 cells with an NFATc1 antisense construct was able to attenuate RANKL-induced osteoclast differentiation. Second, in an article published concomitantly with the original submission of this paper, Takayanagi et al. (56) demonstrated that NFATc1-deficient monocyte/macrophage precursor cells derived from cytokine treatment of NFATc1-null ES cells are unable to undergo osteoclast differentiation in response to RANKL stimulation, indicating that NFATc1 plays an essential nonredundant role in the regulation of RANKL-induced osteoclastogenesis. Furthermore, in agreement with our current findings, this later group also demonstrated that ectopic expression of NFATc1 in bone marrow-derived monocyte/macrophage precursor cells was sufficient to promote the formation of TRAP-positive multinuclear osteoclast-like cells in the absence of RANKL signaling. Taken as a whole, these combined results strongly suggest that the activation of NFATc1 is both necessary and sufficient for the induction of osteoclastogenesis and the acquisition of the mature functional osteoclast phenotype.

The observation that the activation of the NFATc1 transcription factor in osteoclast precursor cells, is, by itself, sufficient to initiate a genetic program that results in the specification of the mature functional osteoclast cell fate serves to define operationally NFATc1 as a potential master regulatory transcription factor for osteoclast differentiation. However, to what extent NFATc1 is directly involved in regulating the expression of the terminal class of genes responsible for specifying the mature osteoclast phenotype remains to be seen, as it is also possible that NFATc1 may merely induce the expression of other transcription factors that are in turn responsible for the expression of osteoclast-specific genes. Resolution of these alternatives will require the identification of the specific NFATc1 target genes in osteoclast precursor cells. In this regard, it is interesting to note that NFATc1 has already been shown to regulate the activity of the TRAP promoter (56) and that putative NFAT binding sites have also been identified in the osteoclast-specific P3 promoter of the calcitonin receptor (66).

Finally, although it appears that the activation of the NFATc1 transcription factor is both necessary and sufficient for osteoclastogenesis, it is important to note that several other transcription factors that affect various aspects of osteoclast development and function have been identified previously. Thus, animals that are genetically deficient in either the ETS family member PU.1, c-Fos, both the p50 and p52 subunits of NF-kB, or both of the helix-loop-helix transcription factors microphthalmia-associated transcription factor and TFE3 have been shown to exhibit an osteopetrotic phenotype (24–26, 67, 68). However, unlike our results with NFATc1, none of these factors alone has been shown to be sufficient to induce osteoclastogenesis independently of other osteoclast-inducing conditions, although overexpression of the c-Fos family member Fra-1 in osteoclast precursor cells has been reported to enhance significantly osteoclastogenesis in the presence of stromal cells, prostaglandin E2, and 1,25(OH)2 vitamin D3 (69). In fact, members of the c-Fos family of proteins are of particular interest because they are subunits of the AP-1 transcription factor complex that is known to be a nuclear partner for NFAT proteins (35). Together, NFAT and AP-1 proteins are known to bind cooperatively to a number of functionally important sites in the promoters of numerous cytokine genes (35). Given the essential roles of both NFATc1 and c-Fos in the regulation of osteoclastogenesis, it is tempting to speculate that they will cooperate directly in the regulation of osteoclast-specific genes. In fact, the interaction between NFATc1 and c-Fos has been shown to be necessary in the regulation of the TRAP promoter (56), although whether NFATc1 interactions with AP-1 are absolutely required for the induction of other osteoclast-specific genes remains to be determined. Although NFATc1 and c-Fos, together with other AP-1 family members, may cooperate in the regulation of certain osteoclast-specific genes, it appears that the functional relationship between NFATc1 and c-Fos in the regulation of osteoclast differentiation is likely to be quite complex because recent studies have also revealed an essential role for c-Fos in the initial RANKL-induced up-regulation of NFATc1 mRNA in osteoclast precursors (56). This observation highlights what will be a major challenge for the future, namely attempting to understand the genetic road map by which each of the transcription factors known to be important for osteoclast differentiation collaborates with each other to ensure the successful completion of the genetic program leading to the acquisition of the mature functional osteoclast cell fate. Nonetheless, whatever the relative functional relationship among these various transcription factors, it is clear from our current data that the calcineurin/NFATc1 signaling pathway plays a prominent role in the regulation of osteoclast differentiation and function, with NFATc1 likely to serve a pivotal role as a critical molecular switch in the regulation of the mature functional osteoclast cell phenotype.

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