Evidence that calcineurin is required for the genesis of bone-resorbing osteoclasts

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Sun L., Peng Y., Zaidi N., Zhu L.-L., Iqbal J., Yamoah K., Wang X., Liu P., Abe E., Moonga BS, Epstein S., Zaidi M. Evidence that calcineurin is required for the genesis of bone-resorbing osteoclasts. Am J Physiol Renal Physiol 292: F285–F291, 2007. First published September 12, 2006; doi:10.1152/ajprenal.00415.2005.—Here, we demonstrate that the Ca2+/calmodulin-sensitive phosphatase calcineurin is a necessary downstream mediator for osteoclast differentiation. Using quantitative PCR, we detected the calcineurin isoforms Aα, Aβ, Aγ (catalytic), and B1 (regulatory) in osteoclast precursor RAW-C3 cells. We found that, although the expression of these isoforms remained relatively unchanged during osteoclast differentiation, there was a profound increase in the expression of their primary substrate for calcineurin, nuclear factor of activated T cells (NFAT)c1. For gain-of-function studies, we incubated osteoclast precursors for 10 min with a calcineurin fusion protein (TAT-calcineurin Aα); this resulted in its receptorless influx into >90% of the precursors. A marked increase in the expression of the osteoclast differentiation markers tartrate-resistant acid phosphatase (TRAP) and integrin β3 followed. In addition, the expression of NFATc1, as well as the alternative substrate for calcineurin, IκBα, was significantly enhanced. Likewise, transfection with constitutively active NFAT resulted in an increased expression of both TRAP and integrin β3 in parallel loss-of-function studies, transfection with dominant-negative NFAT not only inhibited osteoclast formation but also reversed the induction of NFATc1, TRAP, and integrin β3 by TAT-calcineurin Aα. The expression of these markers was also inhibited by calcineurin Aα U1 small nuclear RNA, which significantly reduced calcineurin Aα mRNA and protein expression. Consistent with these observations, we observed a reduction in osteoclastogenesis in calcineurin Aα−/− cells and in osteoclast precursors treated with the calcineurin inhibitors cyclosporin A and FK506. Together, the gain- and loss-of-function experiments establish that calcineurin Aα is necessary for osteoclast formation from its precursor and that this occurs via an NFATc1-dependent mechanism.

Osteoclastogenesis; cyclosporin; nuclear factor of activated T cells

Calcineurin, a Ca2+/calmodulin-dependent protein phosphatase, plays a critical role in transducing Ca2+ signals into cellular responses (22, 23). The heterodimeric protein consists of two subunits: catalytic (A) and regulatory (B) (22). Mammalian calcineurin A has three isoforms (α, β, and γ), which are products of different genes and exhibit ~86% sequence homology. The two isoforms of calcineurin B, in contrast, are highly conserved from yeast to humans (23).

Calcineurin has established roles in T-cell activation, vesicular trafficking, cell growth, apoptosis, neuron depotentiation, muscle development, and cardiac valve formation (20, 27, 33, 37, 44). Calcineurin Aα knockout mice show defective anti-gen-specific T-cell responses (44), cardiac defects (37), and abnormalities in the central nervous system (20, 27). Mechanistically, the enzyme regulates gene transcription by dephosphorylating a family of transcription factors, the nuclear factors of activated T cells (NFATs) (9). In lymphocytes, all four NFAT isoforms are utilized, whereas NFATc1 and NFATc4 are critical transducers in myocytes and neurons, respectively (30, 34). In addition to NFATs, calcineurin can also dephosphorylate the inhibitory subunit of NF-κB, IκB (4).

Calcineurin is potently inhibited by two of the most commonly used immunosuppressants, cyclosporin A and tacrolimus (FK506) (22). The drugs inhibit phosphatase activity through their interaction with distinct domains of calcineurin A (22). Both drugs cause dramatic bone loss in rodents (11, 32). Their administration to humans together with concurrently administered glucocorticoids (for example, after organ transplant) causes acute, rapid, and severe osteoporosis with a high fracture incidence (14, 15). This bone loss is mimicked by the osteoporosis seen in the calcineurin Aα−/− mouse (38). The latter is, at least in part, caused by a marked bone formation defect resulting from inadequate osteoblast differentiation (38). The osteoblastic defect is compounded by the effects of glucocorticoids that inhibit canonical Wnt signaling by increasing the production of the Wnt inhibitors SFRP-1 and dickkopf-1 (5, 6, 28, 29, 36).

Calcineurin and NFATc1 cooperate with the immunoreceptors osteoclast-associated receptor and TREM to play key roles in osteoclast differentiation and the transcription of critical osteoclastic genes (10, 17, 21, 41). Stem cell precursors from NFATc1−/− mice fail to form osteoclasts in response to receptor activator of NF-κB ligand (RANK-L) (40). Overexpression of c-fos, an AP-1 transcription factor, rescues this defect (18). Likewise, NFATc1 overexpression cures the abrogated osteoclastogenesis in c-fos-deficient mice (25). The effects of NFATc1 and c-jun, another AP-1 transcription factor, are cooperative (19). These findings are not unexpected as there is structural evidence for cooperativity between NFAT and AP-1 binding sites (42). In contrast to the positive regulation of osteoclastogenesis, we find that calcineurin overexpression in mature osteoclasts inhibits its bone resorption in the pit assay (39). This makes biological sense because intracellular Ca2+ signals generated by any trigger inhibit bone resorption, whereas they stimulate osteoclast formation (43). It is possible that a switch from NFATc1 to IκB dephosphorylation directs the calcineurin-induced inhibition of bone resorption.

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In this study, we explore the action of calcineurin on osteoclast formation. We first examined the expression of calcineurin isoforms NFAT and IkB during osteoclastogenesis from osteoclast precursors. Gain-of-function experiments used a cell-permeable calcineurin fusion protein, TAT-calcineurin Aα, as well as constitutively active NFAT (NFATC3). Finally, we conducted loss-of-function studies using calcineurin Aα−/− precursors, calcineurin inhibitors, dominant-negative NFAT (NFATM3), and calcineurin Aα U1 small nuclear RNA (snRNA). Collectively, the results show that calcineurin is required for a full osteoclastogenic response; that NFATc1, the primary NFAT isoform, is downstream of calcineurin; and that, in addition to its ability to dephosphorylate all NFATs, calcineurin specifically enhances NFATc1 expression in the osteoclast.

MATERIALS AND METHODS

Cell culture and osteoclast formation assays. Gene expression studies were carried out with RAW-C3 osteoclast precursors that were cultured in α-MEM and FBS (10% vol/vol) with RANK-L (60 ng/ml) for up to 5 days. RAW-C3 is a mouse macrophage subclone of the original RAW264.7 cells, which, in the presence of RANK-L, differentiates into tartrate-resistant acid phosphatase (TRAP)-positive, multinucleated, bone-resorbing osteoclasts (1). The cells thus represent a well-characterized model for studying osteoclastogenesis in vitro in the absence of contaminating osteoblasts. Cells were harvested after 3 and 5 days of culture with RANK-L. Three-day cultures had TRAP-positive mono- and multinucleated osteoclasts as well as precursor cells, whereas, at day 5, the population became predominantly osteoclastic.

In separate experiments, bone marrow cells were isolated from femora and tibias of calcineurin-mutant and wild-type mice in addition to its ability to dephosphorylate all NFATs, calcineurin Aα U1 small nuclear RNA (snRNA). Collectively, the results show that calcineurin is required for a full osteoclastogenic response; that NFATc1, the primary NFAT isoform, is downstream of calcineurin; and that, in addition to its ability to dephosphorylate all NFATs, calcineurin specifically enhances NFATc1 expression in the osteoclast.

Transduction of osteoclast precursors with fusion and mutant proteins. TAT-calcineurin Aα and TAT-hemaglutinin (TAT-HA) were prepared as described previously (13). Osteoclast precursors were incubated with TAT-calcineurin Aα (200 nM) and TAT-HA (200 nM), just once, for 10 min at 37°C and then reincubated in α-MEM for up to 5 days. The cells were then fixed in parafomaldehyde (4%, vol/vol, in PBS; pH 7.4) for 20 min at 20°C and washed with PBS before being exposed to antiserum PP2BA (goat anti-calcineurin Aα antibody; Santa Cruz Biotechnology, Santa Cruz, CA, US) plus a monoclonal anti-TAT antibody or with nonimmune goat or mouse IgG (AbD Advanced Biotechnologies) (all in PBS: 1:100). After 6 h, the coverslips were incubated with donkey FITC-conjugated anti-goat IgG (green) or tetramethylrhodamine isothiocyanate-conjugated antimouse IgG (red) (Jackson Immuno Research Laboratories). An epi-fluorescence microscope (Olympus AX-700) was used to visualize the labeling. Transduction efficiency (>90%) and cytotoxic retention time (up to 5 days) have been demonstrated previously (13).

In separate experiments, RAW-C3 cells were transfected with constitutively activated and NFATM3 using standard protocols with the lipofectamine reagent. To synthesize calcineurin Aα U1 snRNA, the human U1 gene was cloned into pUC19 and designated U1 snRNA stuffer vector (24). PCR-directed mutagenesis was used to construct the modified U1 snRNA anti-calcineurin Aα transcript from endogenous U1 snRNA transcripts. The 5′ primer (mutagenic primer: CTGTCAGACACCACCAAGATCTCATGTCCAGCAGCAGGAGAA) contained the BglII site (GenBank no. J05479). The 3′ primer (AGTGGCAAGCGTTCATGGCAGCAGGTC) was extended beyond the HindIII site, which is downstream of the U1 termination sequence. The 10-bp target sequence was CTGTCA-GACA. The PCR fragments were digested with BglII/HindIII and cloned into the U1 snRNA stuffer vector to produce a set of modified U1 snRNAs, designated U1 anti-calcineurin Aα. DNA sequencing was performed to confirm that the mutations were successfully introduced into the U1 recognition sequence. RAW-C3 cells were cultured in six-well plates (0.9 × 10⁶ per well), grown to ~90% confluence, and cotransfected, using the Fugene6 transfection kit (Roche), with 5 μg of U1 anti-target DNA and 1.5 μg of SV2neo selection vector. The transfected cultures were selected using 400 μg/ml G418.

Quantitative PCR. Total RNA was extracted with the use of a StrataPrep Total RNA mini-prep kit (Stratagene, La Jolla, CA), per the manufacturer’s protocol. Expression levels of various transcripts were determined by quantitative PCR (45). RNA (2 μg) was first reverse transcribed into cDNA. A 1/50 (~10 ng) reverse transcription mixture was utilized for 40-cycle three-step PCR in an ABI Prism 7900HT (Applied Biosystems, Foster City, CA) in 20 mM Tris (pH 8.4), 50 mM KCl, 5 mM MgCl2, 200 μM dNTPs, 0.5× SYBR green I (Molecular Probes, Eugene, OR), 200 nM each primer, and 0.5 U Platinum Taq DNA polymerase (Invitrogen).

The target signal was plotted against the number of PCR cycles, and comparisons between samples were made at the point at which all of the sample reactions were in the linear phase of amplification (the crossing threshold). The intersection of each amplification curve with the threshold yielded a threshold cycle (Ct) value that reflected the relative amount of the original mRNA and cDNA. The initial normalized value in each sample was represented by the ΔCt (target Ct − GAPDH Ct). The results were then calculated as the difference between each normalized reporter signal in a treated sample vs. that in control tubes, yielding the ΔΔCt (treatment ΔCt − control ΔCt). These were expressed as the increase (in fold) at each time point over the respective GAPDH controls (calculated as 2ΔΔCt). Means ± SE were calculated from pooled data from separate experiments, each with triplicate replicates. The method was validated by demonstrating a single band of the expected size for each PCR amplicon by agarose gel electrophoresis (45). Primer quality was checked routinely by obtaining single sharp peaks with melting point association analysis. These tests established the specificity of each PCR reaction, enabling accurate mRNA quantitation.

RESULTS

Expression studies. We first explored whether osteoclast precursors express the calcineurin isoforms. Figure 1A shows a single PCR band for each of the three isoforms of calcineurin A (α, β, and γ) and the two isoforms for calcineurin B (1 and 2) in RAW-C3 osteoclast precursors. We next quantitated the expression of the calcineurin isoforms, the four isoforms of NFATc (1 through 4), and the NF-κB subunits p65, p50, c-Rel, IκBα, and IκBβ during osteoclastogenesis triggered by RANK-L (Fig. 1, B–D). In all cases, results (in fold) are shown with respect to “control conditions,” i.e., at day 0 (without RANK-L), and are normalized to GAPDH. At both days 3 and 5 after induction, there was little change in calcineurin isoform expression, except for a modest approximately twofold elevation in calcineurin Aα and Aγ expression at day 3 (Fig. 1B). However, the expression of NFATc1 was increased by up to 40-fold (Fig. 1C). In contrast, other NFAT isoforms were not affected. No major changes were observed with the NF-κB subunits (Fig. 1D). Together, the results suggest that calcineurin expression remains relatively constant throughout
Transduced cells were grown under differentiating conditions in the presence of RANK-L for 5 days. Figure 2B shows a significant ~2.5-fold increase in NFATc1 and IκBα expression in cells treated with TAT-calcineurin Aα compared with TAT-HA-treated control cells. Inhibition of calcineurin Aα by cyclosporin A (1 μg/ml) abrogated NFATc1 expression (Fig. 2B), again establishing specificity.

In separate experiments, we quantitated the expression of markers of osteoclast differentiation, namely, TRAP and integrin β3. Compared with TAT-HA (control construct) or RANK-L-only cultures, TAT-calcineurin Aα produced a significant increase in the expression of both TRAP and integrin β3 (Fig. 2C). TAT-HA itself did not increase marker expression compared with RANK-L-only cells. Likewise, transfection of cells with NFATΔ5 resulted in an increase in both TRAP and integrin β3 expression compared with control empty vector or RANK-L-only cells at day 5, indicating that NFAT directly regulates osteoclast differentiation (Fig. 2C).

To test whether the effects of NFAT were downstream of calcineurin, osteoclast precursors were cotransduced with TAT-calcineurin Aα and NFATΔ5. The latter almost abolished the stimulation of NFATc1 and TRAP expression at day 5 and significantly attenuated the rise of integrin β3 (Fig. 2C). The results show that calcineurin Aα promotes osteoclast differentiation via NFATc1.

**Gain-of-function studies.** For gain-of-function experiments, osteoclast precursors were incubated with TAT-calcineurin Aα (10 min at 37°C) and stained, following glutaraldehyde fixation, with both anti-TAT (red) and anti-calcineurin Aα (green) antibodies. Cells transduced with vehicle alone showed only anti-calcineurin Aα (green) immune labeling, representing endogenous calcineurin Aα (Fig. 2A, top). However, transduction with TAT-calcineurin Aα resulted in a precise colocalization of the red and green immunostains within the same cells (Fig. 2A, bottom); this is consistent with the influx of exogenously applied fusion protein that was detected by both antibodies.

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calcineurin Aα was likely the main regulator of NFATc1, TRAP, and integrin β3 expression during osteoclastogenesis. It is also possible that cathepsin K expression was regulated by the other isoforms; this requires further testing.

Finally, to definitively establish a role for NFAT in RANK-L-induced osteoclastogenesis, osteoclast precursors were transduced with NFATdn. The first 130 amino acids of NFAT construct abolishes the binding of all NFAT isoforms to cal-

Fig. 2. Gain-of-function studies showing the effects of overexpressing calcineurin Aα (CnAα) as a TAT fusion protein on osteoclast differentiation. A: immunohistochemical detection of TAT-calcineurin Aα with anti-calcineurin Aα antisera (PPB2α, green) and anti-TAT antibody (red) in RAW-C3 cells transduced with TAT-calcineurin Aα fusion protein (200 nM) or vehicle for 10 min at 37°C. Note the overlapping red and green staining (yellow) after transduction, indicating localization of the fusion protein. Retention time of ~5 days and >90% cellular uptake of TAT-calcineurin Aα have been demonstrated previously (13). B: qPCR of RNA isolated from cells transduced with TAT-calcineurin Aα or TAT-hemagglutinin (TAT-HA, control) to demonstrate a stimulation of NFATc1 at 3 days after RANK-L exposure. Cyclosporin A (CsA, 1 μg/ml), a calcineurin inhibitor, was used as a control to abolish all endogenous calcineurin activity. C: qPCR of RNA isolated from cells transduced with TAT-calcineurin Aα with or without dominant-negative NFAT (NFATdn) or separately with constitutively active NFAT (NFATca) or empty vector (EV). Cells were first transfected with NFATdn and then exposed to TAT-HA or TAT-calcineurin Aα (200 nM each) for 10 min. The expression of osteoclast differentiation markers, namely NFATc1, tartrate-resistant acid phosphatase (TRAP), and integrin β3, were studied at day 5 after RANK-L exposure. In B and C, results were normalized to GAPDH (control gene) and RANK-L-only cells. Open bars in C represent untreated controls. Statistics used unpaired Student’s t-test: *P < 0.05 and **P < 0.01.

Fig. 3. Loss-of-function studies. Effect of either calcineurin Aα gene deletion (A and C) or the inhibition of calcineurin enzyme activity by FK506 and calcineurin A (B and D) on TRAP-positive osteoclast formation in Ficoll-purified hematopoietic cell cultures treated with M-CSF and RANK-L for 5 days. In D, wild-type and Aα−/− osteoclast precursors were treated with calcineurin A and FK506 to abrogate all calcineurin activity; results are expressed as percentage of vehicle-treated controls. Solid bars, wild-type controls; open bars, calcineurin-deficient mice. Statistical comparisons, using unpaired Student’s t-test, were made between wild-type and knockout cells (*) or between wild-type groups (§). *P or ^P < 0.05; **P or ^^P < 0.01.
with RANK-L, NFATdn-transfected cells formed fewer osteoclasts than vector-only cells (Fig. 5). At day 5, there was no significant difference between the two groups. Together, the results establish that osteoclast differentiation in response to RANK-L is exerted, at least in part, via calcineurin and NFATc1 expression. Furthermore, the calcineurin-induced enhancement of NFATc1 expression is sharply attenuated by NFATdn, suggesting that NFAT is downstream of calcineurin. There is also a modest increase of calcineurins Aα and Aβ during osteoclastogenesis at which time NFATc1 levels are elevated by ~40-fold. Finally, calcineurin overexpression also stimulates iκBα expression in 5-day cultures. This makes biological sense if osteoclasts, in addition to known effects on nuclear translocation (3).

Molecular regulation of bone resorption. We have shown previously that calcineurin inhibits the resorptive activity of mature osteoclasts (39) and, through this mechanism, likely transduces the effects of intracellular Ca\(^{2+}\) elevations that we have found consistently inhibit osteoclast function (26). By implication, calcineurin might be a downstream transducer of the effects of the high extracellular Ca\(^{2+}\) concentrations generated during bone resorption (35). Activated calcineurin could, in this case, dephosphorylate iκB, an alternate substrate (4), thus preventing the nuclear localization of NF-κB. The latter is expected to reduce resorption by mature osteoclasts. Our present data show, however, that calcineurin stimulates the differentiation of osteoclasts, as does Ca\(^{2+}\) (unpublished observations). We thus speculate that calcineurin has a dual role in regulating bone resorption: it enhances osteoclast differentiation, while inhibiting the resorptive activity of mature cells (39). This hypothesis is internally consistent with histomorphometric data from the calcineurin Aα\(^{-/-}\) mouse showing unaffected resorption surfaces in vivo (38). Precursor cells from this mouse, however, show reduced osteoclastogenic activity in ex vivo cultures. Therefore, it is possible that the inhibition of osteoclast differentiation resulting from calcineurin deletion is balanced by stimulated resorptive activity, with no net effect on resorption surfaces in vivo.

We further hypothesize that NFATc1 is the major substrate for calcineurin during osteoclastogenesis. Consistent with this, there is a dramatic increase in NFATc1 at days 3 and 5. An interesting aspect is that, in addition to activating the nuclear translocation of NFATc1 (3), a known function of calcineurin, the enzyme also stimulates further expression of its substrate, using NFAT itself. Thus overexpression and abrogation of calcineurin, respectively, stimulate and inhibit NFATc1 expression. Furthermore, the calcineurin-induced enhancement of NFATc1 expression is sharply attenuated by NFATdn, suggesting that NFAT is downstream of calcineurin. There is also a modest increase of calcineurins Aα and Aβ during osteoclastogenesis at which time NFATc1 levels are elevated by ~40-fold. Finally, calcineurin overexpression also stimulates iκBα expression in 5-day cultures. This makes biological sense if
mature osteoclasts were to use IκBα as an alternate substrate. In fact, cross-linking studies show that IκBβ competes with NFATc1 for calcineurin binding in vitro (4). The common PEST domain is required for calcineurin-IκB binding, and it is possible that IκBα uses the same mechanism (4). Pathophysiological insights. These observations are relevant to understanding the pathophysiology of the osteoporosis that is seen with calcineurin (activity) inhibitors cyclosporin A and FK506 (11, 14, 15, 32). Either drug, used together with high-dose glucocorticoids, causes profound bone loss in both animal models and humans, in the latter having a fracture risk approaching 70% (8, 14, 15). It has been shown that the osteoporosis, at least at the outset, is of a high-turnover variety, characterized by histomorphometric and biochemical evidence of increased bone resorption (11, 32). This high-turnover osteoporosis could only be explained by a direct and acute acceleration of the resorptive function of mature osteoclasts occurring as a result of not only calcineurin inhibitor action but also effects of glucocorticoids at high doses (12). Alternatively, osteoclast activation may be mediated by T lymphocytes as the bone loss is attenuated in T cell-deficient rats (32). Over time, however, this high-turnover osteoporosis converts to a low-turnover form characterized by low bone formation rates and accompanying decrements in bone resorption. Likely, osteoclastogenesis, and hence bone resorption, becomes impaired with long-standing calcineurin deficiency, as we note in the chronically calcineurin-deprived AxC−/− mouse (38). This mouse also develops a profound bone formation defect due to inhibited osteoblast differentiation and is thus severely osteoporotic. This may in fact be relevant to the development of the adynamic phenomenon posttransplantation with the use of cyclosporin and FK506 where bone loss is mainly due to a profound impairment of bone formation, even in the face of few active osteoclasts (31).

Finally, an interesting clinical paradigm has emerged recently that significantly enhances the importance of our discovery of a key role for calcineurin in bone remodeling. It is now known that the DSCR1 gene, a potent inhibitor of calcineurin activity located on the human chromosome 21, is overexpressed in Down syndrome as a result of trisomy (16). It is believed that such overexpression results in defects in the development of the brain, immune system, heart, and skeleton in these children. Localization of calcineurin to skeletal cells and the demonstration of its function in bone remodeling might therefore be a first step in understanding the molecular pathophysiology of the skeletal phenotype of Down syndrome.

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