Molecular Cloning, Expression and Function of Osteoclastic Calcineurin Aα.

Inhibition of Bone Resorption by TAT-Calcineurin Aα

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Key Words

calcineurin, Ca2+ channel, gene cloning, osteoclast, bone resorption

Short Title
Calcineurin Aα regulates bone resorption

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SUMMARY

This study explores the role of the calmodulin- and Ca^{2+}-sensitive phosphatase, calcineurin A, in the control of bone resorption by mature osteoclasts. We first cloned full-length calcineurin Aα and Aβ cDNA from a rabbit osteoclast library. Sequence analysis revealed a ~95% and 86% homology between the amino acid and nucleotide sequences, respectively, of the two isoforms. The two rabbit isoforms also showed significant homology with the mouse, rat and human homologs. In situ RT-PCR showed evidence of high levels of expression of calcineurin Aα mRNA in freshly isolated rat osteoclasts. Semi-quantitative analysis of staining intensity revealed no significant difference in calcineurin Aα expression in cells treated with vehicle versus those treated with the calcineurin (activity) inhibitors cyclosporin A (8 x 10^{-7} M) and FK506 (5 x 10^{-9} and 5 x 10^{-7} M). We then constructed a fusion protein comprising calcineurin Aα and TAT, a 12-amino acid-long Arg-rich sequence of the HIV protein. Others have previously shown that the fusion of proteins to this sequence results in their receptor-less transduction into cells, including osteoclasts. Likewise, unfolding of the TAT-calcineurin Aα fusion protein by shocking with 8 M urea resulted in its rapid influx, within minutes, into as many as 90% of all freshly isolated rat osteoclasts, as was evident on double immunostaining with anti-calcineurin Aα and anti-TAT antibodies. Pit assays performed with TAT-calcineurin Aα-positive osteoclasts revealed a concentration-dependent (10 to 200 nM) attenuation of bone resorption in the absence of cell cytotoxicity or changes in cell number. TAT-hemaglutinin did not produce significant effects on bone resorption or cell number. The study suggests that (a) the 61 kD protein phosphatase, calcineurin Aα, can be effectively transduced into osteoclasts using the TAT-based approach, and (b) the transduced protein retains its capacity to inhibit osteoclastic bone resorption.
INTRODUCTION

Maintenance of skeletal integrity depends upon a precise balance between bone formation and resorption. An absolute or relative increase of resorption over formation results in bone loss. Bone is removed by osteoclasts and is rebuilt by osteoblasts as part of the bone remodeling process. The activity of both osteoclasts and osteoblasts is regulated by precise molecular signals some of which are sensitive to changes in cytosolic Ca\(^{2+}\) (50). The osteoclast in particular is exposed to high millimolar extracellular concentrations of Ca\(^{2+}\) during resorption (40). It has an extracellular Ca\(^{2+}\) sensor thought to be a type II ryanodine receptor expressed at the plasma membrane (47, 30, 49).

Calcineurin is the only serine/threonine protein phosphatase sensitive to both Ca\(^{2+}\) and calmodulin that plays a critical role in coupling Ca\(^{2+}\) signals to cellular responses (22, 23, 41). The calcineurin heterodimer comprises of one catalytic and one regulatory subunit (subunits A and B, respectively); the latter is highly conserved from yeast to humans (16). The three known isoforms of mammalian calcineurin A (α, β and γ) are products of different genes and exhibit ~86% sequence homology (GenBank Accession No. J05479, M81483, and NM_008915 respectively). Calcineurin Aα is widely distributed (24, 8, 18, 25, 28) and has established roles in T cell activation, vesicular trafficking, cell growth, apoptosis, neuron depotentiation, muscle development, and cardiac valve formation. We recently provided preliminary evidence that calcineurin Aα, expressed in osteoclasts, plays a role in the regulation of bone resorption (4).

Here, we propose to understand more fully the function of calcineurin A in bone resorption using a complement of molecular and cellular approaches. Firstly, we cloned full-length cDNAs for the calcineurin A isoforms, α and β. We then utilized in situ RT-PCR to demonstrate mRNA expression in freshly isolated mature osteoclasts. Using the same technique, we examined, in a semi-quantitative manner, changes in expression of calcineurin
Aα in response to two known inhibitors of calcineurin activity, cyclosporin A and tacrolimus (FK506). Both inhibitors are known to inhibit calcineurin’s phosphatase activity, but may have downstream effects on calcineurin Aα expression (37, 3). Both cyclosporin A and FK506 also cause profound bone loss in vivo both in animals and man (7, 4). However, the precise mechanism of their action on bone cells remains unclear.

To evaluate the function of calcineurin in osteoclastic bone resorption, we developed a method through which we were able to transduce most of the relatively sparse population of cells that we isolate freshly from neonatal rats. The technique involved creating a fusion protein comprising calcineurin Aα and TAT, a 12 amino acid arginine-rich sequence of the HIV protein (32). A control protein, TAT-hemaglutinin (HA) was also similarly synthesized and purified. TAT fusion proteins, particularly those that have been unfolded by 8M urea treatment, have been shown to traverse cell membranes rapidly (46). At least two proteins, β3 and rho A, have been successfully transduced into osteoclasts (1, 11). The mechanism of the effect of TAT is unclear, although current evidence suggests that TAT-assisted cellular permeation of proteins is receptor-independent (46).

We first detected high efficiency transduction through the use of an anti-TAT antibody. We then examined the function of freshly isolated osteoclasts transduced with TAT-calcineurin Aα, with TAT-HA as control, using the traditional pit assay in which the resorption of bone and number of resorbing cells is quantitated by simple morphometry. We found that TAT-calcineurin Aα inhibited osteoclastic bone resorption, while TAT-HA did not. Both fusion proteins did not affect cell number. Overall therefore, the study provides compelling molecular evidence (a) that the calcineurin A isoforms α and β are expressed in osteoclasts, and (b) that calcineurin Aα inhibits bone resorption.
MATERIAL AND METHODS

Cloning of calcineurin Aα and Aβ isoforms

A rabbit osteoclast cDNA library constructed in the λZAP II expression vector containing 1 x 10^10 independent clones, kindly provided by Professor Kumegawa (Saitama, Japan), was used for PCR amplification (44, 21). The oligonucleotide primers for calcineurin Aα and Aβ were designed based on analyses of the nucleotide sequences of rat, mouse, human and bovine cDNA as described previously (4). Their primer sequences were: calcineurin Aα, forward: 5’-CGACAGGAAAAAAACTTGCTGGAT-3′ (424-447), reverse: 5’-GTTTGGCTTTTCTGTACATG-3’ (1094-1075) (GenBank Accession No. D90035); calcineurin Aβ, forward: 5’-AACCATGATAGAAGTAGAAGCT-3’ (294-315), reverse: 5’-CACACACTGCTGGATAGTTATAA-3’ (865-843) (Genbank Accession No. D90036). The coding regions of the calcineurin Aα and Aβ cDNA fragments were then amplified by PCR in a final volume of 50 µl containing 0.1 µl of rabbit osteoclast cDNA library (1 x 10^7 independent clones), 1 µl of each oligonucleotide (50 µM), and 1 µl (5 U) of AmpliTaq (Perkin Elmer, Foster City, CA). The GeneAmp PCR System 2400 (Perkin Elmer, Foster City, CA) was programmed as follows: 94°C for 5 minutes, then 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 40 seconds. The PCR products were separated by agarose gel electrophoresis. The 670 and 570 bp fragments of calcineurin Aα and Aβ, respectively, were isolated from excised gel slices using a gel purification kit from Qiagen (Valencia, CA) and ligated into EcoRV-cut pBluescript II SK+ vector (Stratagene, La Jolla, CA). The resulting plasmids pBS-CNAα670 and pBS-CNAβ570 were then transformed into competent DH5α cells. The sequences of both inserts were confirmed by sequence analysis and used as probes for library screening.

To obtain the full-length calcineurin Aα and Aβ, the 670-bp and 570 bp fragments were labeled with [α^32P]-dCTP (3000Ci/mmol) respectively (NEN Life Science, Boston, MA) using
the Redprime Random Prime Labeling Kit (Amersham Pharmacia Biotech, Piscataway, NJ). DNA manipulation was performed using standard protocol as described by Sambrook et al. (36). Approximately 1 x 10^7 plaques of the rabbit osteoclast cDNA library were screened initially with the probes of calcineurin Aα and β. Plating with independent clones made two replica filters. After SDS-alkali treatment, Tris neutralization, and cross-linking of nucleic acids to nylon membrane using a GS Gene Linker UV chamber (Bio-Rad, Hercules CA), the filters were hybridized overnight at 42°C with labeled probe in a solution containing formamide (50%, v/v), 6x SSC, 5x Denhardt’s, SDS (0.5%, w/v), and denatured fragmented salmon sperm DNA (0.1 mg/ml). After a high stringency wash at 68°C for 1 hour, the filters were subjected to autoradiography for 20 hours at –70°C. Positive recombinant plaques were purified from phage lysates according to the Lambda ZAP II library’s instruction (Stratagene, La Jolla, CA). The cDNA clones were confirmed by Southern blot and PCR analysis. The positive clones were then sequenced and compared with those of calcineurin Aα and Aβ from human, mouse and rat.

**In situ RT-PCR Cytoimaging of Freshly Isolated Osteoclasts**

The method has been described in detail in our previous publications that contain the primer sequences for cathepsin K and GAPDH (2, 43). Osteoclasts were isolated from neonatal rat long bones and cultured on glass coverslips in Medium 199 with Earle’s salts (6.6 mM Na₂CO₃, M199-E) for 6 hours, following which they were fixed with paraformaldehyde (4% v/v) in PBS for 20 minutes at 4°C. After two washes with cold PBS, the fixed cells were treated with 0.2 N HCl for 20 minutes at 20°C and washed with DEPC-water (Sigma). Cells were then treated with proteinase-K (5 µg/ml in 10 mM-Tris-HCl, pH 8) for 15 minutes at 37°C followed by cold paraformaldehyde (4% v/v) for 30 minutes at 4°C. Prior to being air-dried, the cells were dehydrated by sequential immersions in ethanol solutions, 70, 80, 90 and 100% (v/v), for 1 minute at each concentration. The samples were then incubated overnight (37°C) with 1500
U/ml RNase-free DNase I (Boehringer Mannheim, Indianapolis, IN) to remove genomic DNA. First-strand cDNA was synthesized by incubating cultures with 50 µl RT mixture (1 mM dNTP, 0.01 M DTT, 400 nM reverse primer in DEPC-water, and 14 U/µl SuperscriptII) for 60 minutes at 4°C. The samples were then treated separately with 50 µl PCR mixture containing 0.2 mM dNTP, 1X PCR buffer, 2.5 mM MgCl₂, 0.1 U/µl Taq polymerase, 400 nM forward and reverse primers, 10 µM digoxigenin-labelled-11-dUTP (Boehringer Mannheim). Each sample was then covered gently with an AmpliCover disc ensuring the absence of air bubbles. The GeneAmp In Situ PCR System 1000 (Perkin Elmer) was programmed as follows: 94°C for 4 minutes; 40 cycles; 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes.

Incorporated DIG-11-dUTP in the PCR product was detected by an alkaline phosphatase-conjugated anti-DIG antiserum and AP substrates, 4-nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl-phosphate (BCIP) using a DIG Nucleic Acid Detection Kit (Boehringer Mannheim) per manufacturer’s protocol. Negative controls, in which primers were omitted, were run in parallel. Messenger RNA expressing cells stained dark purplish brown, whilst negative controls did not stain. We then performed an analysis of the staining intensity using a blinded observer as described previously (2, 43). Osteoclasts were scored on a scale from 0 to 3 (no staining to intense staining). The results were then plotted as a frequency histogram. This allowed us to determine the proportion of cells that lie in a certain intensity range. This analysis was utilized to examine the effect of incubating osteoclasts with cyclosporin A (8 x 10⁻⁷ M) or FK506 (5 x 10⁻⁹ and 5 x 10⁻⁷ M) or appropriate vehicle on gene expression in separate experiments.

**Synthesis, Purification and Transduction of TAT-calcineurin Aα**

We inserted a 42 bp double stranded oligomeric nucleotide encoding the 12 amino acid TAT protein transduction domain flanked by glycine residues (YGRKKRRQRRRG) and BamHI
and \textit{XhoI} restriction endonuclease recognition sites at the 5' and 3' ends, respectively, into pRSET A vector (Invitrogen, Carlsbad, CA). This generated the plasmid, pTAT, which was then transformed into competent DH5α cells. Transformants were selected on LB agar plates containing 100 µg/ml ampicillin. Colonies expressing ampicillin resistance were screened for the presence of the pTAT recombinant plasmid by \textit{BamHI/XhoI} restriction analysis and the sequence of insert was confirmed by sequencing analysis. Complementary DNA for full-length calcineurin Aα was inserted in-frame into the \textit{XhoI/EcoRI}-cut pTAT expression vector. The resulting plasmid, pTAT-CNAα, contains 6-histidine tag followed by the 12 amino acid TAT transduction domain. The pTAT-HA vector, containing an 11 amino acid TAT domain was a kind gift from Professor Abou Amer (Washington University, St. Louis, MO).

The expression constructs pTAT-CNAα and pTAT-HA were transformed into \textit{E.coli BL21 (DE3) pLysS} cells, and allowed to grow at 30°C in 1 l SOB medium containing 100 µg/ml ampicillin for 4 hours to mid-log phase. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM. The incubation was continued for another 3 hours. Cells (1.2 g wet weight) were harvested by centrifugation at 4000 x g for 20 minutes. The cell pellet was resuspended in 10 ml of buffer A (6 M guanidine hydrochloride, 0.1 M NaH\textsubscript{2}PO\textsubscript{4}, 0.01 M Tris, pH 8.0) and stirred for 1 hour at room temperature followed by sonication on ice until turbid. After centrifugation at 12,000 x g for 15 minutes, the supernatant containing crude extracts was applied to a Ni-NTA purification column (Qiagen) and washed with ten bed volumes of buffer A, five bed volumes of buffer B (8 M urea, 0.1 M NaH\textsubscript{2}PO\textsubscript{4}, 0.01 M Tris, pH 8.0), and 10 bed volumes of buffer C (8 M urea, 0.1 M NaH\textsubscript{2}PO\textsubscript{4}, 0.01 M Tris pH 6.3) plus 0.2 M NaCl. The resin-bound TAT-calcineurin Aα or TAT-HA fusion proteins were subsequently eluted with buffer C containing 0.25 M imidazole. Urea was removed by dialysis against PBS in a volume of 2 liters for 8 hours at 4°C. A total of 4 and 8 mg recombinant TAT-calcineurin Aα and TAT-HA proteins, respectively, were obtained and stored at –70°C.
**Immunocytochemistry and Confocal Imaging**

Freshly isolated osteoclasts (as above) were incubated in α-MEM containing 10% FBS for 24 hours. Serum was removed and incubation was continued for a further 2 hours. The cells were incubated with TAT-calcineurin Aα (200 nM) for 10 minutes at 37°C, fixed in paraformaldehyde (4%, v/v, in PBS, pH 7.4) for 20 minutes at 20°C, incubated with pre-cooled ethanol/acetic acid (2:1), and washed with PBS (Gibco-BRL). The cells were then exposed to polyclonal goat anti-calcineurin Aα antiserum (PP2BAα; Santa Cruz, Santa Cruz, CA) or non-immune goat IgG or antiserum PP2BAα plus a mouse monoclonal anti-TAT antibody (ABI Advanced Biotechnologies, Columbia, MD) (for co-localization studies) (all in DMEM, 1:100). After 6 hours incubation, the coverslips were rinsed gently with PBS, drained, and re-incubated with donkey FITC-conjugated anti-goat IgG (green) or with both anti-goat IgG and TRITC-conjugated anti-mouse IgM (red) (for co-localization experiments) (in PBS for 60 minutes) (Jackson ImmunoResearch Laboratories, West Grove, PA). The coverslips were then washed gently and drained. An epifluorescence microscope (Olympus AX-700) was used to visualize the staining using FITC and rhodamine filters, as appropriate.

**Bone Resorption Assay**

The crude osteoclast suspension isolated in Medium 199-H from neonatal (24 to 48 hour-old) rat long bone was dispersed directly on devitalized bone slices (6, 10, 13, 31). The cells were allowed to settle for 30 minutes following which each slice was washed in Medium 199-E (with 10% FBS v/v) to remove contaminants. The bone-osteoclast cultures were further allowed to incubate in the same medium at enable attachment, and then incubated with various concentrations of TAT-calcineurin Aα or TAT-HA (10 to 200 nM) for 10 minutes at 37°C. The cells were washed again and allowed to incubate overnight at 37°C in humidified 5% CO₂ (pH 6.9) following which the slices were fixed in 10% glutaraldehyde and stained for tartrate-resistant acid phosphatase (TRAP) using a kit (Kit 386A; Sigma Chemical, St. Louis, MO).
Multinucleated osteoclasts were counted and the slices were then bleached with NaOCl (5 minutes) before air drying and staining for toluidine blue to allow for visualization of the osteoclastic excavations (pits). The number of pits was determined by light microscopy. Each experiment was performed with osteoclasts obtained from three animals with five or six bone slices per treatment. The number of pits or osteoclasts per bone slice was expressed as means ± SEM. Student’s unpaired t test with Bonferroni’s Correction for Inequality was used to analyze the effect of treatment, which was considered significant at $p < 0.05$. Note that at pH 6.9, resorptive activity is maximal (10, 13).

RESULTS

Two positive cDNA clones were obtained by screening $1 \times 10^7$ clones of the rabbit osteoclast cDNA library using the 670 bp calcineurin Aα and 570 bp calcineurin Aβ cDNA coding region of the respective PCR fragments as probes. Figure 1 shows the full-length osteoclast calcineurin Aα and Aβ cDNA coding region as well as the predicted amino acid sequences. There was 68% and 78% similarity between the cDNA and amino acid sequences of calcineurin Aα and Aβ, respectively (GenBank Accession No. AF541960 and AF541961).

In addition to the coding region sequence shown in Figure 1, we have also obtained the full sequence of the cloned cDNA. Notably, nucleotide sequence analysis of the 1836 bp cloned cDNA fragment of the calcineurin Aα gene revealed a 1536 nucleotide long coding region. This encoded a 511 amino acid protein ($M_r$ - 56 kDa), and contained 396 and 284 nucleotides representing the 5’- and 3’-UTRs, respectively. The 1575 bp coding-region of the Aβ gene encoded a 525 amino-acid protein ($M_r$ - 58 kDa), and contained 36 and 1621 bps of 5’- and 3’-UTRs.

The cDNA sequence of osteoclastic calcineurin Aα was 94%, 93%, 93% and 93% similar to corresponding full-length cDNA coding region sequences of the human (GeneBank:
BC025714), mouse (J05479), rat (X57115) and bovine (U33868) homologs, respectively. The cDNA sequence of osteoclast calcineurin Aβ was 97%, 95% and 94% similar to corresponding full-length cDNA coding region sequences of the human (NM_021132), mouse (M81483), and rat (NM_017042) homologs, respectively. On the other hand, no significant homology was found between the sequence of the inserts and any other sequence in the GenBank database.

Having cloned full-length calcineurin Aα, we next explored whether it was expressed in freshly isolated mature osteoclasts. The same primers, as used above, were employed in in situ RT-PCR experiments using a cytoimaging technique described before (2, 43). Figure 2A shows light micrographs of histostained osteoclasts after RT-PCR. Panel a shows an untreated osteoclast in an experiment in which primers were omitted. Panels b and c show intense brown staining demonstrating the expression of two control genes: cathepsin K (cell-specific control) and GAPDH (housekeeping gene). Panel d shows an osteoclast staining for calcineurin Aα mRNA following vehicle treatment. Panel e to g show similarly intense calcineurin Aα mRNA histostaining osteoclasts that had been treated with 8 x 10^-7 M cyclosporin A (e), 5 x 10^-9 M FK506 (f), or 5 x 10^-7 M FK506 (g).

On visual examination, we found no differences in the overall staining pattern in treated osteoclasts compared with untreated cells. We then performed a semi-quantitative analysis of staining intensity using a method modified from that reported by Adebanjo et al (2). Figure 2B shows osteoclasts staining for calcineurin Aα mRNA were assessed by a blinded observer who assigned an intensity level to the staining as a number between 0 and 3 (no staining to intense staining). Osteoclasts that underwent in situ RT-PCR incubated with primers, but without treatment (a, n = 16 cells), showed a normal (Gaussian) distribution of their assigned scores. Unlike what we have seen before with our studies with interleukin-6 (2), the data did not become significantly skewed when osteoclasts were treated with 8 x 10^-7 M cyclosporin A (b, n = 25 cells), 5 x 10^-9 M FK506 (c, n = 11 cells) or 5 x 10^-7 M FK506 (d, n = 25 cells). This suggested
that the calcineurin activity inhibitors, cyclosporin A and FK506, did not significantly alter calcineurin Aα gene expression.

We next explored whether calcineurin could affect the resorptive function of mature osteoclasts. It is difficult to transfect or virally infect mature resorbing osteoclasts with genes encoding proteins of interest, mainly because of the sparse number and limited life span of freshly isolated cells. We therefore utilized a novel TAT transduction method (see above) for delivering the calcineurin Aα protein into osteoclasts. This involved the initial synthesis of a TAT-calcineurin Aα fusion protein in E. coli. We constructed a plasmid, pTAT-CNAα, that contained the 12 amino acid TAT protein transduction domain (YGRKKRRQRRRG), the cloned calcineurin Aα gene, and N-terminal of 6-histidine tag (Figure 3a). The plasmid was transformed into BL21 cells that were induced with IPTG. The resulting protein was purified with a Ni-NTA affinity column. We similarly synthesized and purified TAT-HA as a control fusion protein. Western blotting of supernatants with the anti-calcineurin Aα antibody or anti-HA antibody showed intense bands, molecular sizes ~63 and 12 kDa, which corresponded to the TAT-calcineurin Aα and TAT-HA fusion proteins, respectively.

Isolated osteoclasts were then transduced with 200 nM TAT-calcineurin Aα by incubation at 37°C for 10 minutes. The cells were then co-stained with a polyclonal anti-calcineurin Aα antiserum (green) and a monoclonal anti-TAT antibody (red). Without transduction, osteoclasts were found to immunostain only with the anti-calcineurin Aα antiserum (green only), not with anti-TAT antibody, confirming the presence of calcineurin Aα protein in untransduced osteoclasts (Figure 4a to 4c). However, after transduction, intense and a mostly overlapping pattern (orange to yellow) of red and green staining was noted (Figure 4d to 4f). Immunodetection by both anti-calcineurin Aα and anti-TAT antibodies strongly suggested influx of the applied TAT-calcineurin Aα fusion protein.
We next examined the bone resorptive function of osteoclasts transduced with TAT-calcineurin Aα. As we cannot visualize staining in osteoclasts settled on bone, we carried out immunostaining experiments (as above) in parallel with our resorption assays for consistency of our methodology and to ensure the uptake of the TAT fusion protein. Following a 10-minute incubation at 37°C, osteoclasts previously settled on devitalized bone slices, were allowed to incubate further for 18 hours. Figure 5 shows a highly significant, concentration-dependent (10 to 200 nM), reduction in the number of pits per slice, with no change in the number of cells per slice (p values in legend). In contrast, the control protein TAT-HA (10 to 200 nM) did not significantly inhibit bone resorption or change cell number. The latter indicates that the effect of TAT-calcineurin Aα on osteoclast resorptive function was not due to cell toxicity, although subtle effects on cell viability cannot be excluded.

DISCUSSION

The expression of calcineurin isoforms in the osteoclast is not unexpected. Firstly, osteoclasts are unique in handling high Ca²⁺ loads, both extracellularly and intracellularly (50). One would therefore expect this cell to possess a phosphatase that was responsive to changes in cytosolic Ca²⁺: calcineurin fits that role perfectly. Secondly, and perhaps more important is the likely critical role of calcineurin in controlling gene transcription in the osteoclast, a cell that passes through several stages of differentiation before acquiring a bone resorptive phenotype (45). Finally, during resorption, osteoclasts actively secrete both acid and proteolytic enzymes (48). The underlying process of vesicular trafficking has been shown to be sensitive to calcineurin in other cells, such as synaptic neurons (26).

Our cloning and sequencing of two calcineurin isoforms, Aα and Aβ, from a cDNA library constructed previously from pure rabbit osteoclast preparations by Kumegawa and
colleagues (44) provides definitive evidence for their osteoclastic expression. Furthermore, using *in situ* RT-PCR, we show that the calcineurin Aα mRNA is expressed in mature osteoclasts that are capable of resorbing bone. We also show using a specific anti-calcineurin Aα antibody that the protein is expressed in osteoclasts.

Clearly, the expression of calcineurin in mature osteoclasts raises the question of its possible function in bone resorption. We provide direct evidence that TAT-calcineurin Aα, the cell-permeant calcineurin isoform, inhibited bone resorption by isolated osteoclasts, while the control TAT-HA protein did not. Osteoclast number did not change with either fusion protein thereby excluding cytotoxicity and apoptosis. Previous apparently paradoxical observations on the inhibition of bone resorption by cyclosporin A, a calcineurin inhibitor, do continue to exist (4). However, it is known that cyclosporin A interacts with other cellular targets independently of calcineurin; this may explain inhibition of bone resorption with the drug (39).

Several mechanisms nevertheless underscore the anti-resorptive action of TAT-calcineurin Aα in the absence of cell toxicity. Firstly, it is possible that calcineurin might directly dephosphorylate proteins involved in vesicular trafficking in response to changes in cytosolic Ca\(^{2+}\) occurring as a result of bone resorption (42). Proteins, such as dynamin, may be critical targets in inhibiting acid secretion and enzyme release (26). Secondly, calcineurin may trigger the redistribution of osteoclast integrins in response to Ca\(^{2+}\) transients through a direct effect, as has been shown for other cells (27, 34). While this effect is particularly relevant to the osteoclast, a direct molecular interaction between α\(_v\)β\(_3\), the main osteoclast integrin, and calcineurin has not yet been established.

Thirdly, a longer term and possibly more physiologically relevant response could occur through effects on gene transcription exerted through the traditional NFATc signaling pathway used by calcineurin in lymphocytes and cardiac cells (12). In lymphocytes, activation of
calcineurin by calmodulin or Ca\(^{2+}\) results in transactivation of several critical genes, including
the GM-CSF, interleukins 2, 3, 4 and 5, CD40, and the Fas genes (33, 19, 38, 12). While these
responses are mediated by the transcription factors NFATc1 through 4 (12), cardiac endothelial
cell growth and hippocampal neuronal stimulation involve NFATc1 and NFATC4, respectively
(17, 35). We are unclear as to which NFATc isoform is involved in calcineurin effects on
osteoclasts. In addition, from our unpublished studies on myoblastic cells and other published
evidence, we can speculate additional dephosphorylation targets for calcineurin in the
osteoclast, notably IκBβ, NFκB, and MeF (5, 14, 29).

It is likely that critical genes for Ca\(^{2+}\) release channels including IP\(_3\) receptors (IP\(_3\)Rs) and
ryanodine receptors (RyRs) that are widely expressed in the osteoclast are potential targets for
osteoclastic calcineurin. We have shown that the overexpression of calcineurin A\(\alpha\) in
myoblastic C2C12 cells results in dramatic increases in the expression of RyR-1 (unpublished).
We have also shown in the same cell type that oxidative stress resulting from mitochondrial
DNA deletion, for example, is associated with the enhanced expression of both RyR-1 and
calcineurin A\(\alpha\) (5). Thus, there is a clear direct relationship between the expression of
calcineurin A\(\alpha\) and target RyR genes. In addition, inhibitors such as FK506 decouple the
molecular interaction between RyRs and calcineurin (20, 9). Should the relationship between
calcineurin and RyR expression hold in the osteoclast, it would be of special relevance to the
function of this cell. Osteoclasts express high levels of type II RyR uniquely at their plasma
membrane (49). We have provided evidence that this surface expressed RyR-II plays a critical
role in extracellular Ca\(^{2+}\) sensing, a process by which an osteoclast monitors changes in ambient
Ca\(^{2+}\) levels and transduces intracellular Ca\(^{2+}\) signals during bone resorption (50, 49). A high
intracellular Ca\(^{2+}\) level during bone resorption could potentially, through the activation of
calcineurin, result in elevated RyR-II expression. Admittedly speculative, this may be a positive
feedback mechanism through which the sensitivity of the osteoclast to changes in extracellular
Ca\(^{2+}\), exerted via RyR expression, is maintained during resorption.
While the physiological relevance of calcineurin in bone resorption, and the molecular mechanisms thereof remain issues for further investigation, this study clarifies that calcineurin does not alter its own expression. From our in situ RT-PCR studies, albeit semi-quantitative, it is clear that the two inhibitors, cyclosporin A or FK506, did not affect the expression of the calcineurin Aα gene. In other words, calcineurin gene expression is not regulated by its phosphatase activity. Likewise, to our knowledge, such regulation has not been documented in other cells. It is also unlikely that TAT-mediated transduction is affected by cyclosporin A or FK506, as these drugs are not known to affect TAT delivery into cells.

An interesting clinical paradigm has emerged recently that significantly enhances the importance of our discovery of calcineurin in the osteoclast. It is now known that the DSCR1 gene, a potent inhibitor of calcineurin activity located on the human chromosome 21, is overexpressed in Down’s syndrome as a result of trisomy (15). 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 in a skeletal cell, such as the osteoclast, and a prediction of its function in skeletal remodeling, might therefore be a first step in understanding the molecular pathophysiology of the skeletal defects in Down’s syndrome (12).

Finally, this study further documents the use of the TAT-transduction system as a reliable means of transducing mature osteoclasts with proteins with high efficiency. At least two reports have similarly used TAT to transduce the mutated form of ΙκΚβ and the small GTP-binding protein Rho, respectively, into osteoclasts (11, 1). The system, developed initially by Dowdy and colleagues, establishes a new paradigm for protein transduction in sparse populations of cells, such as osteoclasts, without the need to infect, transf ect or microinject (46).
In conclusion, we have documented the existence of the Ca^{2+}/calmodulin phosphatase calcineurin in osteoclasts and demonstrated its role as an inhibitor of bone resorption. We have also shown that calcineurin Aα can be effectively delivered into osteoclasts as a functionally active protein through its fusion to TAT, an Arg-rich sequence derived from the HIV protein. The study paves the way for future investigations to study calcineurin signal transduction in osteoclasts, using similar cell-permeant constructs of calcineurin and its signaling molecules, such as the NFATc isoforms.

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REFERENCES


LEGRNDST TO FIGURES

Figure 1

The cDNA and amino acid sequences of calcineurin (CN) Aα and Aβ cloned from a cDNA library that was constructed previously from freshly isolated rabbit osteoclast preparations. Gaps have been introduced to maximize homology.

Figure 2

(A) In situ reverse transcriptase polymerase chain reaction (RT-PCR) performed on freshly isolated osteoclasts using primers constructed for calcineurin Aα, cathepsin K (Cath K, cell specific control gene), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, housekeeping gene). Cells were also treated with calcineurin inhibitors, cyclosporin A (CsA) or FK506. (B) Semi-quantitative estimates of staining intensity shown in frequency histograms. Staining intensity was graded as described in the Materials and Methods section by an independent blinded observer who scored the intensity from zero (no staining) to 3 (intense staining) in three experiments. The data were analyzed statistically for skews and shifts were considered significant if p < 0.01.

Figure 3

Synthesis and purification of TAT-calcineurin Aα fusion protein. (a) Construction of plasmid pTAT (2.95 kb) by inserting the TAT sequence and the calcineurin Aα coding region cDNA. (b) Western immunoblot of either a crude extract of BL21 (DE3) pLysS cells transformed with pTAT-calcineurin Aα or following its purification on a Ni-NTA column. The left-most lane represents control cells that were not transformed. An anti-calcineurin Aα antiserum (PP2BAα) was used to immunostain the blots.
Figure 4

Double immunostaining of isolated osteoclasts with a polyclonal goat anti-calcineurin Aα antiserum (PP2BAα) and monoclonal mouse anti-TAT antibody. FITC-labeled anti-goat IgG (green) and TRITC-conjugated anti-mouse IgM (red), respectively, were used as secondary antibodies. Panels a to c represent an osteoclast that was incubated with vehicle; hence the absence of anti-TAT (red) staining in panel b. Panel a shows the endogenous expression of calcineurin Aα. Panels d to f represent a cell that was incubated with 200 nM TAT-calcineurin Aα for 10 minutes at 37°C. Both anti-calcineurin Aα (green, panel d) and anti-TAT (red, panel e) staining were noted that were superimposable in the merged panel f.

Figure 5

Effect of incubation with TAT-hemaglutinin (HA) or TAT-calcineurin Aα (10, 100 and 200 nM) for 10 minutes at 37°C on osteoclastic bone resorption (number of pits per slice, panels a and c) and osteoclast number (number of cells per slice, panels b and d). Statistics by Student’s t-test with Bonferroni’s Correction for Inequality. Significance, $p < 0.01$. 
Figure 3

(a) Schematic representation of the expression plasmid pTAT. The plasmid contains the TATA box (ATG-His6-TAT), CNAα, and restriction sites such as XhoI, EcoRI, and BstBI. The plasmid size is 2.95 kb.

(b) Western blot analysis showing the expression of CNAα in control, TAT-CNAα, and purified TAT-CNAα samples. The protein at 63 kDa is specifically recognized as CNAα.
Figure 5

(a) Number of pits/slice vs. TAT-calcineurin Aα (nM)

(b) Number of cells/slice vs. TAT-calcineurin Aα (nM)

(c) Number of pits/slice vs. TAT-HA (nM)

(d) Number of cells/slice vs. TAT-HA (nM)