JOURNAL OF BONE AND MINERAL RESEARCH
Volume 16, Number 3, 2001

Human Osteoclast Cathepsin K Is Processed Intracellularly Prior to Attachment and Bone Resorption

ROBERT A. DODDS,[1] IAN E. JAMES,[1] DAVID RIEMAN,[1] REINA AHERN,[1] SHING MEI HWANG,[1] JANICE R. CONNOR,[1] SCOTT D. THOMPSON,[2] DANIEL F. VEBER,[2] FRED H. DRAKE,[1] STEPHEN HOLMES,[3] MICHAEL W. LARK,[1] and MAXINE GOWEN[1]

ABSTRACT

Cathepsin K is a member of the papain superfamily of cysteine proteases and has been proposed to play a pivotal role in osteoclast-mediated bone resorption. We have developed a sensitive cytochemical assay to localize and quantify osteoclast cathepsin K activity in sections of osteoclastoma and human bone. In tissue sections, osteoclasts that are distant from bone express high levels of cathepsin K messenger RNA (mRNA) and protein. However, the majority of the cathepsin K in these cells is in an inactive zymogen form, as assessed using both the cytochemical assay and specific immunostaining. In contrast, osteoclasts that are closer to bone contain high levels of immunoreactive mature cathepsin K that codistributes with enzyme activity in a polarized fashion toward the bone surface. Polarization of active enzyme was clearly evident in osteoclasts in the vicinity of bone. The osteoclasts apposed to the bone surface were almost exclusively expressing the mature form of cathepsin K. These cells showed intense enzyme activity, which was polarized at the ruffled border. These results suggest that the in vivo activation of cathepsin K occurs intracellularly, before secretion into the resorption lacunae and the onset of bone resorption. The processing of procathepsin K to mature cathepsin K occurs as the osteoclast approaches bone, suggesting that local factors may regulate this process. (J Bone Miner Res 2001;16:478–486)

Key words: cathepsin K processing, osteoclast, cytochemistry, ruffled border

INTRODUCTION

OSTEOCLASTS ARE specialized multinucleated cells that resorb bone. The resorbing osteoclast creates an extracellular compartment, which it maintains at an acid pH via a vacuolar H^+-adenosine triphosphatase (ATPase) and into which it secretes hydrolytic enzymes.[1–4] The acidity of the compartment results in demineralization of the matrix and provides an optimal pH for certain proteolytic enzymes,[1,3,5] which ultimately degrade the demineralized matrix. An intensively convoluted membrane, the ruffled border,[2] lines the resorption lacuna that forms beneath a recruited osteoclast. Newly synthesized lysosomal enzymes, packaged into transport vesicles, are transported to this membrane, where they are secreted into the underlying compartment. Two reports have shown that osteoclasts in vitro remove the organic and inorganic degradation products by endocytosis at the ruffled border membrane, with subsequent transcytosis (vesicular) through the cell to the apposing free membrane, and liberation into the extracellular space.[6,7] This process is inhibited by a nonselective cysteine protease inhibitor *trans*-epoxysuccinyl-*l* leucylamido-(4-guanidino) butane (E64).[7]

Lysosomal cysteine protease(s) plays a key role in bone matrix resorption.[8–12] An osteoclast selective cysteine pro-

[1]Department of Bone and Cartilage Biology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania, USA.
[2]Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania, USA.
[3]Department of Molecular and Cellular Immunology, SmithKline Beecham Pharmaceuticals, Harlow, United Kingdom.

tease cathepsin K has been identified[13–18] that has high sequence homology to cathepsins S and L. In situ hybridization, Northern blot, and immunolocalization studies indicated that human cathepsin K was expressed abundantly in osteoclasts but that cathepsins B, L, and S were expressed at relatively low or negligible levels.[13,14,16,19]

Cathepsin K has a low pH optimum and degrades many bone matrix proteins including type I collagen, osteopontin, and osteonectin.[20–22] Selective human cathepsin K inhibitors have been described that potently inhibit osteoclast resorption both in vitro and in vivo.[23–25] Patients with pycnodysostosis, a disease characterized by abnormal bone remodeling,[26–30] have mutations in the cathepsin K gene.[31,32] Furthermore, mice with a null mutation in the cathepsin K gene develop osteopetrosis of the long bones and vertebrae.[33,34] Histological evaluation of resorption sites from the bones of −/− mice revealed fully differentiated osteoclasts apposed to small regions of demineralized bone, suggesting that the cathepsin K–deficient osteoclasts are capable of demineralizing the extracellular matrix but are unable to degrade adequately the demineralized matrix.[34]

Purified cathepsin enzyme studies, using synthetic fluorogenic substrates,[20,35] have shown that benzyloxycarbonyl-Phe-Arg-(4-methyl)coumaryl-amide (Cbz-Phe-Arg-MCA) and Cbz-Leu-Arg-MCA are the favored substrates for both cathepsin K and cathepsin L. In contrast, cathepsin K does not cleave the cathepsin B selective substrates Cbz-Arg-Arg-MCA and Cbz-Ala-Arg-Arg-MCA.[20,35] Cytochemical analysis of the localization and activity of cysteine proteases in tissue sections are possible using similar synthetic substrates that contain the leaving group 4-methoxy-2-naphthylamide (4MβNA).[36] During the assay, performed on cryostat sections, the cleaved naphthalamine is subsequently postcoupled with Fast Blue BB to form a quantifiable red precipitate within the cell. The modified quantitative cytochemical assay described here uses the cathepsin K substrates, Cbz-Phe-Arg-4MβNA, and Cbz-Leu-Arg-4MβNA, and comparison was made using the cathepsin B substrates Cbz-Arg-Arg-4MβNA and Cbz-Ala-Arg-Arg-4MβNA. Cryostat sections of human fetal bone (tibia, 19-week gestation) and osteoclastoma were used to identify and characterize cathepsin K activity. Fetal bone contains abundant resorbing osteoclasts at the site of endochondral bone formation; however, nonresorbing osteoclasts are rare. In contrast, osteoclastoma tissue contains abundant nonresorbing osteoclasts distant from populations adjacent to or attached to membranous bone, representing an ideal tissue to investigate lysosomal cathepsin processing.

The present study was designed to investigate whether cathepsin K in situ is secreted into the osteoclast resorption lacunae in its inactive (proform) or mature form.

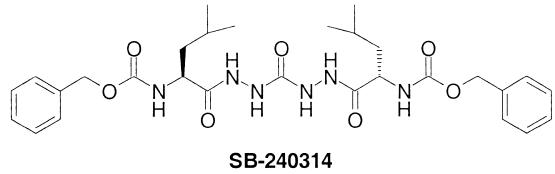

FIG. 1. Structure of SB 240314.

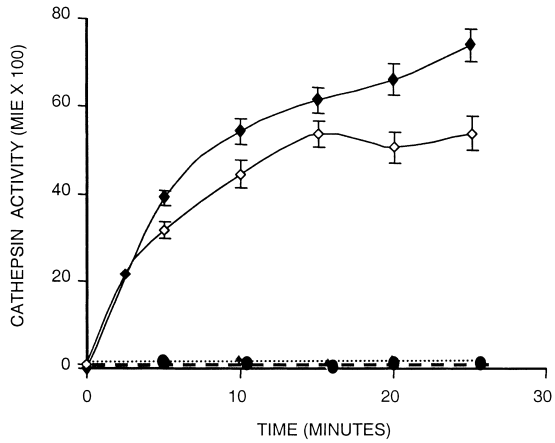

FIG. 2. Measurement of cathepsin activity with time of reaction. Assays were conducted at pH 5.5 using Z-Leu-Arg-4MβNA (15 mM) as substrate. Activity is expressed as MIE × 100 ± SEM. Osteoclasts within human osteoclastoma section (open diamonds) and osteoclasts within fetal human bone sections (closed diamonds) were evaluated. No activity was recorded in fetal bone osteoclasts with the cathepsin B substrates Z-Arg-Arg-4MβNA (dotted line) and Z-Ala-Arg-Arg-4MβNA (hatched line). The cathepsin B assays were carried out at 12 mM substrate concentration at pH 6.0.

MATERIALS AND METHODS

Tissue processing

Human fetal bone and kidney (Anatomical Gift Foundation, Laurel, MD, USA) and osteoclastoma tissue (Jefferson Hospital, PA, USA) were obtained (with informed consent) at the time of surgery and frozen as described previously.[37] Adult kidney samples were obtained from the Anatomical Gift Foundation (Laurel, MD, USA). Multiple serial sections from each tissue block (5 μm) were cut on a Hacker cryostat (Hacker Instruments, Inc., Fairfield, NJ, USA), equipped with a finely polished tungsten-tipped steel knife, and flash-dried onto glass slides. Serial sections were processed for enzyme cytochemistry (cysteine protease), immunolocalization, and in situ hybridization as described in the following section. For in situ hybridization studies, TESPA-coated (3-aminopropyltriethoxy silane; Sigma Chemical Co., St. Louis, MO, USA) glass slides were used.

Cysteine protease activity

Cryostat sections of whole tissue were assayed for protease activity by a suitably modified azo coupling procedure derived from the cathepsin B assay described by Van Noorden et al.[36] For the detection of cathepsin K, sections were incubated at 37°C for 10–15 minutes in the following reaction medium (total volume, 12 ml): 5 ml encapsin HPB (Hammond, Cerestar, IN, USA) added to 6 ml of 40% polypeptide (dissolved in 100 mM phosphate buffer, pH 5.3; Sigma), the cathepsin K substrate (dissolved in 1 ml

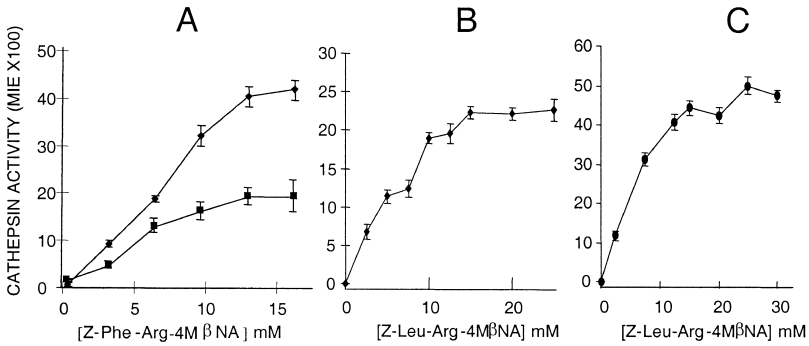

FIG. 3. (A) The effect of increasing concentrations of Z-Phe-Arg-4MβNA on the cathepsin activity of osteoclasts within sections of human osteoclastoma (closed diamonds) and proximal convoluted tubule cells within sections of human kidney (closed squares). Assays were conducted at pH 5.5 for 15 minutes. (B and C) The effect of increasing concentrations of Z-Leu-Arg-4MβNA on the activity of (B) osteoclastoma and (C) fetal osteoclast cathepsin activity; assays were conducted at pH 5.5 for 10 minutes. Activity is expressed as MIE $\times$ 100 $\pm$ SEM.

DMSO), Cbz (Z)-Leu-Arg-4MβNA (15 mM) or Z-Phe-Arg-4MβNA (12 mM; Bachem, King of Prussia, PA, USA), and 2.5 mM EDTA. Subsequently, the sections were post-coupled at room temperature for 10 minutes with 0.25 mg/ml Fast Blue BB (Sigma) dissolved in phosphate-buffered saline (PBS). The sections were then rinsed in PBS. The sections were finally rinsed with 100 mM copper sulfate for 10 minutes at room temperature. The final reaction product is a deep red precipitate. Two cathepsin B substrates also were profiled: Z-Arg-Arg 4MβNA and Z-Ala-Arg-Arg-4MβNA (Bachem).

Specificity was assessed by using the nonselective cysteine protease inhibitor *trans*-epoxysuccinyl-*l* leucylamido-(4-guanidino) butane (E64; Sigma), the aspartate protease inhibitor pepstatin A (Sigma), the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Sigma), and a selective irreversible inhibitor of cathepsin K SB 240314 (Fig. 1).[24] For SB 240314, the $k_{obs}/[I]$, M^{-1} s^{-1} for inhibition of cathepsin K, B, and L are 3.1×10^6, 1.3×10^3, and 5.8×10^4, respectively; the K_i for cathepsin S was 11 nM (i.e., >1000-fold selective for cathepsin K vs. cathepsin B, L, and S).

The reaction product was measured on a per cell basis using a Vickers M85 scanning and integrating microcytophotometer,[38] with a mask that encompasses a single cell (ensuring that the optical density of a unit area of reaction product was measured in each cell), a $\times$40 objective at 550 nm, and with a flying spot of a 0.5-μm diameter in the plane of the section. The individual flying spot measurements of "relative extinction" was integrated by the microcytophotometer and summed to give the relative integrated extinction or absorption over the area scanned. By suitable calibration (with a filter of known absolute absorbance), the absolute mean integrated extinction (MIE) was determined for each cell. Thirty cells were measured in triplicate sets of human osteoclastoma (osteoclasts), 19-week gestation fetal femoral bone (osteoclasts), and kidney (proximal convoluted tubule cells). Randomized measurements are recorded blind at distinct sites for each section. Results are presented as MIE $\times$ 100 $\pm$ SEM or expressed as percent of control.

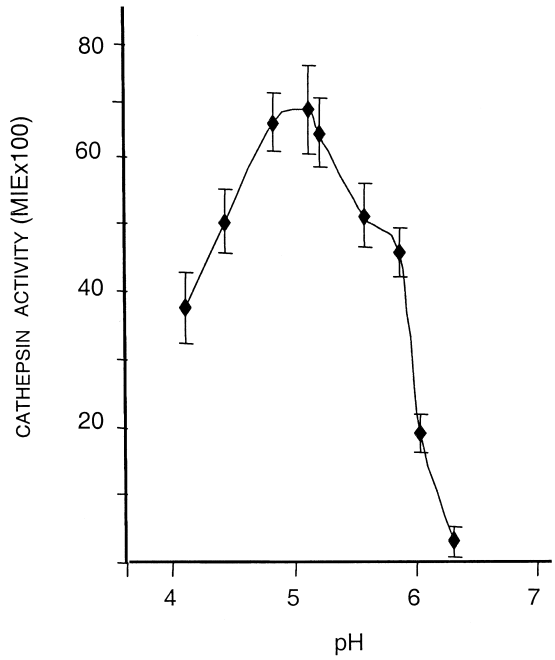

FIG. 4. Measurement of optimum pH. The assay was conducted on sections of fetal tibia using 15 mM Z-Leu-Arg-4MβNA for 10 minutes. Activity is expressed as MIE $\times$ 100 $\pm$ SEM.

Immunolocalization

The generation and characterization of the murine monoclonal antibodies CE5 (procathepsin K) and 10C7 (mature cathepsin K) is described elsewhere.[39,40] 10C7 shows no cross-reactivity with procathepsin K or cathepsins L, B, D, or S. A panel of soft tissues (cryostat sections) known to have abundant levels of cathepsin B, L, or S (e.g., liver, skin, muscle, and lung macrophages) also were stained with 10C7 and CE5 and no expression was observed. For immunolocalization, an alkaline phosphatase-based immunoenzymatic technique was used according to the manufacturers protocol (Dako, Carpenteria, CA, USA). A new fuchsin-based substrate system was used to localize antibody binding and a red precipitate indicates positive reactivity. No expression was observed in isotype-matched negative controls.

In situ hybridization

A complementary DNA (cDNA) clone (pBluescript SK) containing the coding region of human cathepsin K was

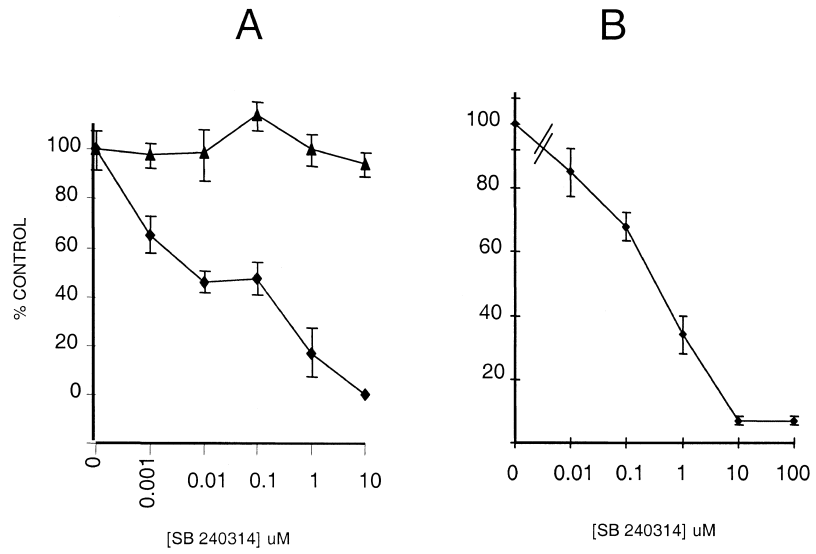

FIG. 5. Inhibition of osteoclast cathepsin activity by SB 240314. (A) Assays were conducted with Z-Phe-Arg-4MβNA at pH 5.5 for 15 minutes on sections of osteoclastoma (diamonds) and adult kidney (triangles). SB 240314 had no effect on cathepsin activity in kidney proximal convoluted tubule cells (cathepsin L). Results presented as percent of control ± SEM. (B) Assay conducted with Z-Leu-Arg-4MβNA as substrate on sections of fetal tibia. Results are presented as percent of control ± SEM.

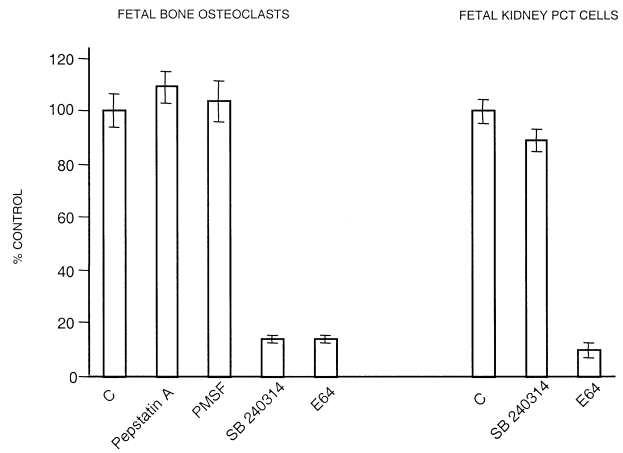

FIG. 6. Effects on nonselective protease inhibitors on cathepsin activity in sections of fetal bone and fetal kidney proximal convoluted cells (PCT). Pepstatin (10 μM, aspartyl protease inhibitor) and PMSF (10 μM, serine protease inhibitor) had no effect on cathepsin activity in fetal bone osteoclasts. In contrast SB 240314 (1 μM) and E64 (1 μM) potently inhibited osteoclast cathepsin activity. In contrast to E64, SB 240314 had no effect on fetal kidney cell cathepsin activity. Assays were conducted with Z-Leu-Arg-4MβNA at pH 5.5 for 10 minutes. Results are presented as percent of control ± SEM.

obtained from an osteoclast library. cDNA templates were linearized and then transcribed from the T3 or the T7 promoter to generate sense and antisense probes, respectively. Riboprobes were prepared using the Promega (Madison, WI, USA) in vitro transcription kit with [^{35}S]thiocytosine triphosphate (CTP; Amersham, Arlington Heights, IL, USA). After transcription, cDNA templates were digested with RQ1 RNAse-free DNAse I (Promega) and unincorporated nucleotides were removed by centrifugation through Quick Spin Sephadex G-50 columns (Boehringer-Mannheim, Indianapolis, IN, USA). RNA transcripts with a specific activity in excess of 10^8 cpm/mg were used for hybridization. In situ hybridization was performed as previously described.[37]

RESULTS

To validate true enzyme activity cytochemically, the assay should show substrate dependancy, linearity with time of reaction, and the appropriate pH optimum of the enzyme. Optimization (to attain maximal activity) and confirmation of cathepsin activity was performed on cryostat sections of human osteoclastoma tissue (containing zones of intramembranous bone remodeling), fetal bone (19-week gestation), and adult (70 years old, diseased) and fetal kidney (19-week gestation); kidney proximal convoluted tubule cells contain no cathepsin K but do contain abundant cathepsin B and L expression.[13,37] The fetal kidney blocks were in excellent condition compared with the adult kidney (rejected for transplant purposes) and were thus used for the selective versus nonselective inhibitor studies.

Substrate specificity, linearity with time, and pH profile

For each experiment, representative data are presented from one of four experiments. The selective cathepsin K cytochemical substrates Z-Phe-Arg-4MβNA and Z-Leu-Arg-4MβNA were excellent substrates for osteoclast cathepsin activity. The activity was linear with time of reaction up to 20 minutes (Fig. 2), and the pattern of activity was comparable with either substrate. No activity was observed in osteoclastoma osteoclasts (not shown) or fetal human osteoclasts with the cathepsin B substrates Z-Arg-Arg-4MβNA or Z-Ala-Arg-Arg-4MβNA (Fig. 2), consistent with the negligible expression of cathepsin B messenger RNA (mRNA) and protein.[13] Chondrocytes and macrophages showed cathepsin activity in sections of fetal bone using these cathepsin B substrates (data not shown). Osteoclast cathepsin activity was dose dependent with both substrates (Fig. 3). Proximal convoluted tubule cells in

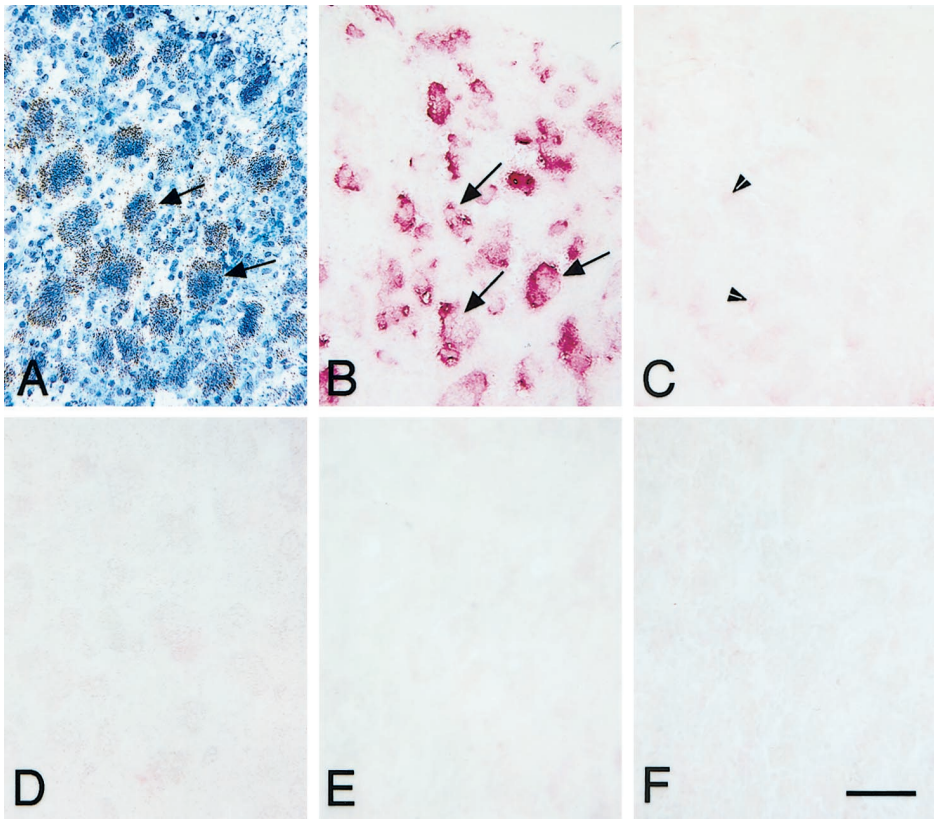

FIG. 7. Expression of pro- and mature cathepsin K, cathepsin K mRNA, and cathepsin activity in serial sections of osteoclastoma. (A) Section hybridized with the cathepsin K antisense probe shows osteoclasts (arrows) with intense and selective levels of cathepsin K mRNA. Counterstained with methylene blue. (B) Serial section reacted with the procathepsin K monoclonal antibody (CE5). This population of osteoclasts shows intense levels of procathepsin K (arrows). (C) Serial section reacted with the mature cathepsin K monoclonal antibody (10C7). This population of osteoclasts shows no mature cathepsin K (arrows). (D) Serial section reacted for cathepsin activity using the substrate Z-Phe-Arg-4MβNA. (E) Serial section reacted for cathepsin activity using the substrate Z-Leu-Arg-4MβNA. (F) Isotype matched negative control for panel B (bar = 80 μm).

sections of human kidney also showed cathepsin activity (cathepsin L) with Z-Phe-Arg-4MβNA (Fig. 3) and Z-Leu-Arg-4MβNA (not shown).

For both substrates (Z-Leu-Arg-4MβNA shown as representative), the osteoclast cathepsin activity was maximal in the acidic pH range from 5.0 to 5.5 (Fig. 4), consistent with the published pH optimum of isolated cathepsin K.

Confirmation of cysteine protease activity

Specificity was addressed by the use of the nonselective cysteine protease inhibitor E64, the aspartate protease inhibitor pepstatin A, the serine protease inhibitor PMSF, and a selective irreversible inhibitor of cathepsin K SB 240314. SB 240314 (Fig. 5) and E64 (Fig. 6) potently inhibited osteoclast cathepsin activity in both osteoclastoma and fetal bone sections. Pepstatin A (10 μM) and PMSF (10 μM) were inactive (Fig. 6). SB 240314 did not inhibit cathepsin activity in proximal convoluted kidney cells (Figs. 5 and 6). In contrast, the nonselective cysteine protease inhibitor E64 potently inhibited kidney cathepsin activity (Fig. 6). These data suggest that the cathepsin activity in the kidney is cathepsin L.

Nonresorbing osteoclasts express cathepsin K mRNA and procathepsin K protein but do not show cathepsin activity

Serial cryostat sections of human osteoclastoma tissue (blocks from 5 patient samples were selected, either containing or not containing discrete sites of bone formation and remodeling) were probed with specific antibodies to either procathepsin K, mature cathepsin K, reacted for cathepsin activity, or hybridized with a cathepsin K riboprobe to localize sites of cathepsin K mRNA expression. Multiple sections were examined from the different osteoclastoma blocks. The representative sites highlighted in Figs. 7 and 8 were selected from the same section for each probe/activity described previously. Osteoclasts that were distant from bone (Fig. 7) showed intense expression of cathepsin K mRNA (Fig. 7A, homogeneously localized), contained procathepsin K (Fig. 7B, CE5 positive) but negligible levels of mature cathepsin K (Fig 7C, 10C7 negative). This mature cathepsin K–negative population of cells also did not show any cathepsin activity (Figs. 7C and 7D). Equivalent results were shown in osteoclastoma tissues that contained no zones of intramembranous ossification (data not shown).

Cathepsin K processing occurs before osteoclast attachment and bone resorption and correlates with enzyme activity detected cytochemically

Sites of osteoclastoma tissue were selected in which osteoclasts were adjacent to zones of intramembranous ossification (osteoclasts apposed to bone were evident but rare at this site). Osteoclasts that were closest to zones of ossification in the osteoclastoma tissue showed low expression of the proform of the enzyme (CE5 negative, arrows in Fig. 8A), and high expression of the mature form (10C7 positive,

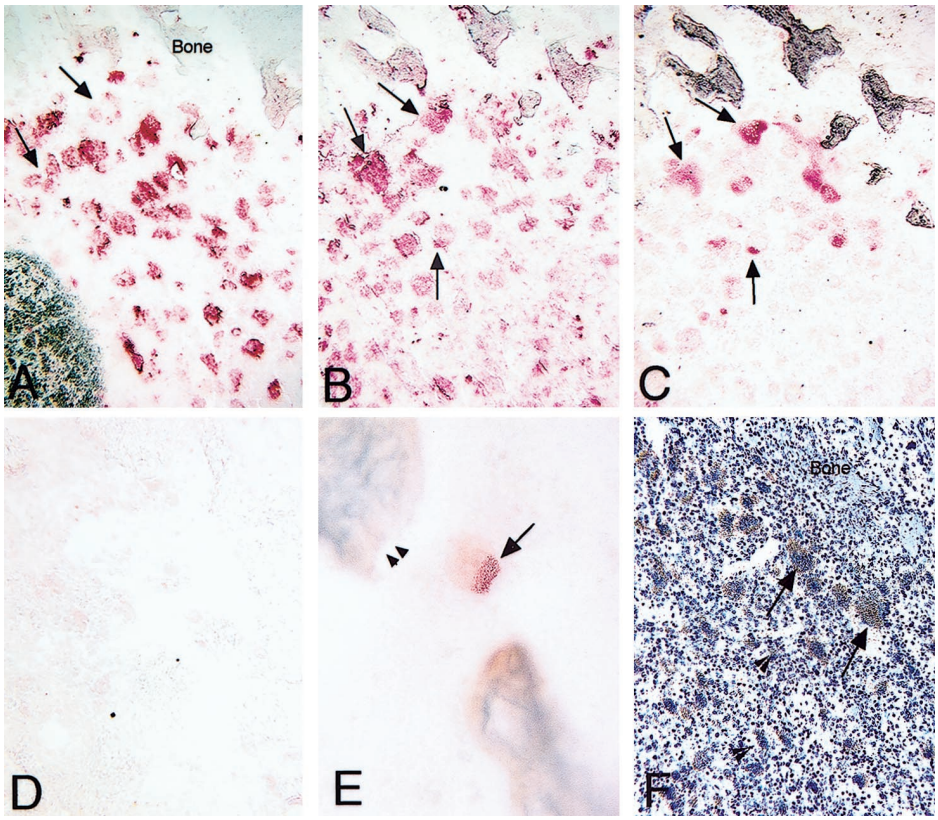

FIG. 8. Section of osteoclastoma that contains zones of intramembranous ossification; serial sections were reacted with an antibody to (A) procathepsin (CE5), (B) mature cathepsin K (10C7), and (C) reacted for cathepsin activity (Z-Phe-Arg-4MβNA). Cathepsin K processing (arrows) clearly occurs before osteoclast attachment and resorption. (D) Isotype matched negative control for panel B. (E) An osteoclast (fetal bone) situated between bone (arrowheads) already shows polarized enzyme activity (Z-Phe-Arg-4MβNA). (F) Serial section to panel C. Cathepsin K mRNA expression in osteoclasts (arrows) adjacent to bone. Osteoclasts distant from bone were smaller and are indicated by arrowheads. Section counterstained with methylene blue (bar = 100 μm).

arrows at top of Fig. 8B). The expression of mature cathepsin K directly correlated with enzyme activity detected cytochemically (Fig. 8C). Polarization of active enzyme was evident in osteoclasts near bone in osteoclastoma (Fig. 8C, arrows) and fetal bone (Fig. 8E). Although the polarity of cathepsin K activity in Fig. 8C (middle arrow) appears opposite to the nearby bone (top of figure), this could be explained by polarity toward bone deeper (and thus hidden) within the block of osteoclastoma tissue. Cathepsin K mRNA expression appeared highest in the larger osteoclasts adjacent to bone (Fig. 8F).

At sites of bone remodeling in sections of osteoclastoma (Figs. 9A–9C) osteoclasts resorbing bone almost exclusively expressed the mature form of cathepsin K that colocalized with intense enzyme activity polarized at the osteoclast-bone interface. In contrast to nonresorbing osteoclasts, cathepsin K mRNA expression was highly variable in the resorbing osteoclast population, ranging from a negligible to a moderate signal (Fig. 9D). In sections of fetal bone reacted for cathepsin K activity, high power views of osteoclast resorption sites revealed a distinct pattern of polarization (Figs. 9E and 9F).

DISCUSSION

During osteoclast-mediated bone resorption, the organic constituents of bone are degraded by lysosomal enzymes, which are secreted directionally into the resorbing compartment.[4] Ultrastructural studies have shown that osteoclasts do not contain high concentrations of phagocytic structures such as secondary lysosomes. Instead, hydrolytic enzymes are found in elements of the exocytic pathway[41] reflecting a high biosynthetic activity. Cysteine protease(s) plays a key role in bone matrix degradation.[8–12] A number of groups have identified a novel cysteine protease, cathepsin K, that appears to be selective for the osteoclast and plays a pivotal role in bone matrix degradation.[13,34] Localization studies showed that although human cathepsin K was expressed abundantly in osteoclasts, cathepsins B, L, and S were expressed at very low levels or were not detectable.[13,14,16,19] Furthermore, transgenic mice lacking cathepsin L activity had a normal bone phenotype.[42] Cytochemical analysis of cathepsin activity in sections of cathepsin K knockout mouse bone revealed that, in contrast to wild-type (+/+) osteoclasts, −/− osteoclasts showed no cathepsin activity, confirming that cathepsin K is the predominant cysteine protease of the osteoclast (unpublished observations).

In vitro assays, using isolated human osteoclasts seeded onto bone chips, have revealed activated cathepsin K in osteoclast resorption lacunae and trails.[25] In mammalian cells, cysteine proteases are synthesized as latent precursors, and can either be secreted as proenzymes or be transported via mannose-6-phosphate receptors to lysosomal compartments where they can be processed to the mature active enzyme, to either play an intracellular role or be secreted.[43] The pH underneath the ruffled border of the osteoclast is

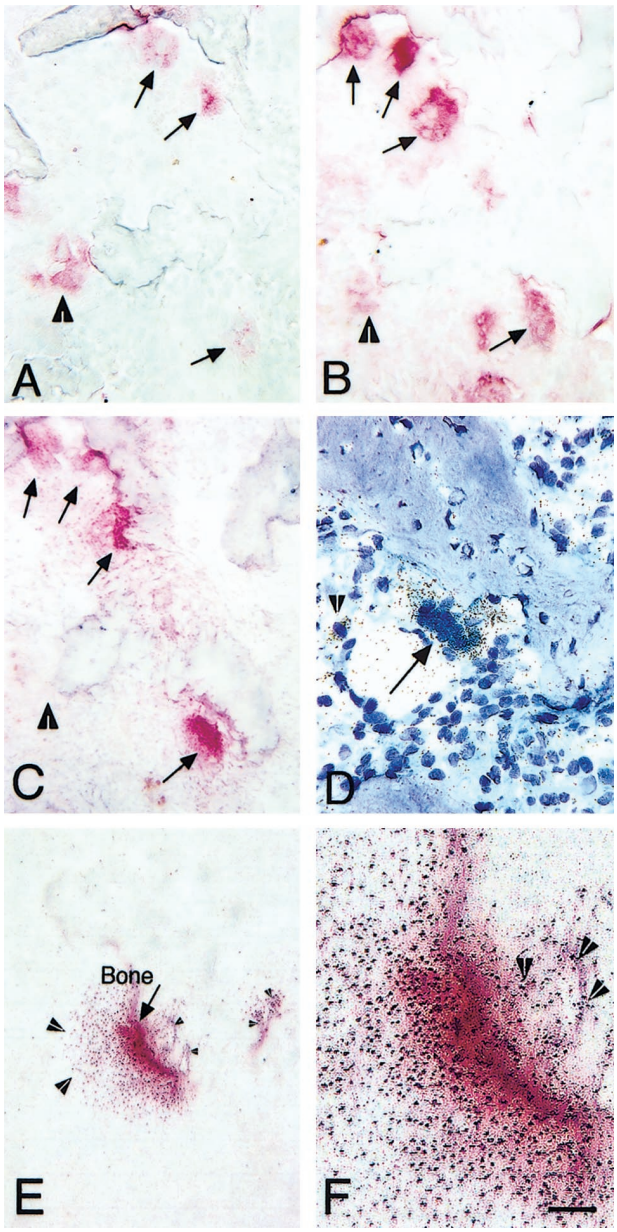

FIG. 9. Sections of osteoclastoma that contain zones of (remodeling) intramembranous ossification; serial sections were immunoreacted with antibodies (A) CE5, (B) 10C7, (C) reacted for cathepsin activity (Z-Phe-Arg-4MβNA), or (D) hybridized with the cathepsin K antisense probe. Osteoclasts are indicated (arrows and arrowheads). (E) An osteoclast (arrowheads) resorbing mineralized bone in a section of fetal bone reveals intense polarized activity at the osteoclast-bone interface. Note the gradient of activity observed in vesicles spanning from the apical surface (large arrowheads) to the ruffled border. Processes embedded into the bone also show cathepsin activity (small arrowheads). Section reacted for cathepsin activity using the Z-Phe-Arg-4MβNA substrate. (F) High power view of panel E highlights the osteoclasts processes (arrowheads; bar = 40 μm).

approximately pH 4.0.[5,44] However, it is speculated that the pH at the zone closest to bone is more neutral, because of the buffering capacity of dissolved bone salts.[21] Stability studies suggest that the neutral pH zone of the resorption lacunae is the site of cathepsin K and interstitial collagenase activity,[21,45] but that the activation of procathepsin K occurs in the low pH environment by either cleavage by another protease or by an autocatalytic process.[20,21,46] To address this issue, we developed a microcytophotometric assay to localize and quantify osteoclast cathepsin K activity in sections of human bone. The distribution of enzyme activity was then compared with the pattern of immunoreactive pro- or mature cathepsin K protein expression.

Sections of osteoclastoma, which included abundant osteoclasts distant from bone formation sites, showed intense expression of cathepsin K mRNA, high levels of procathepsin K, but negligible levels of mature cathepsin K. This population of osteoclasts showed no cathepsin activity. We further show that as osteoclasts approach bone (in both osteoclastoma and fetal bone), there is processing of the proenzyme to mature cathepsin K that directly correlated with enzyme activity detected cytochemically. Similarly, in both bone tissues, polarization of cathepsin K was observed in osteoclasts before attachment to bone, suggesting that the activation and polarization of cathepsin K is regulated by bone and/or local factors. The osteoclasts apposed to bone (in both fetal and osteoclastoma) almost exclusively expressed the mature form of cathepsin K and showed intense enzyme activity, again polarized at the ruffled border. High-power microscopy of fetal bone revealed a gradient of activity from the apical surface of the osteoclast to the ruffled border, consistent with lysosomal trafficking to the ruffled border. One consistent observation noted in sections of osteoclastoma tissue was that osteoclasts close (not attached) to bone appeared larger and had higher cathepsin K mRNA levels when compared with osteoclasts more distant from bone. In contrast, osteoclasts attached to bone showed highly variable expression. It remains to be determined whether this variable expression pattern reflects transcriptional regulation of the cathepsin K gene potentially at different stages of osteoclast function.

The use of a sensitive cathepsin K cytochemical assay in conjunction with immunolocalization and in situ hybridization confirmed that cathepsin K activation occurs intracellularly, before secretion into the resorption lacunae and the onset of bone resorption. We further showed that cathepsin K processing and polarization occurs as the osteoclast approaches bone, implying that local factors in the microenvironment may regulate these events.

ACKNOWLEDGMENTS

We thank Dr. Richard Lackman from the Jefferson Hospital, Philadelphia, PA, USA, for supplying the osteoclastoma tissue and Heather McClung for technical support.

REFERENCES

1. Blair HC, Teitelbaum SL, Ghiselli R, Gluck S 1989 Osteoclastic bone resorption by a polarized vacuolar proton pump. Science **245:**855–857.

2. Baron R, Neff L, Brown W, Courtoy PJ, Louvard D, Farquhar MG 1988 Polarized secretion of lysosomal enzymes: Co-distribution of cation-independent mannose-6-phosphate receptors and lysosomal enzymes along the osteoclast exocytic pathway. J Cell Biol **106:**1863–1872.
3. Vaananen HK, Karhukorpi EK, Sundquist K, Wallmark B, Roininen I, Hentunen T, Tuukkanen J, Lakkakorpi P 1990 Evidence for the presence of a proton pump of the vacuolar H(+)-ATPase type in the ruffled borders of osteoclasts. J Cell Biol **111:**1305–1311.
4. Hall TJ, and Chambers TJ 1996 Molecular aspects of osteoclast function. Inflamm Res **45:**1–9.
5. Silver IA, Murrills RJ, Etherington DJ 1988 Microelectrode studies on the acid microenvironment beneath adherent macrophages and osteoclasts. Exp Cell Res **175:**266–276.
6. Salo J, Lehenkari P, Mulari M, Metsikko K, Vaananen HK 1997 Removal of osteoclast bone resorption products by transytosis. Science **276:**270–273.
7. Nesbit SA, Horton MA 1997 Trafficking of matrix collagens through bone-resorbing osteoclasts. Science **276:**266–269.
8. Hill PA, Buttle DJ, Jones SJ, Boyde A, Murata M, Reynolds JJ, Meikle MC 1994 Inhibition of bone resorption by selective inactivators of cysteine proteinases. J Cell Biochem **56:**118–130.
9. Debari K, Sasaki T, Udagawa N, Rifkin BR 1995 An ultrastructural evaluation of the effects of cysteine-proteinase inhibitors on osteoclastic resorptive functions. Calcif Tissue Int **56:**566–570.
10. Delaisse JM, Eeckhout Y, Vaes G 1984 In vitro and in vivo evidence for the involvement of cysteine proteinases in bone resorption. Biochem Biophys Res Commun **125:**441–447.
11. Lerner UH, Grubb A 1992 Human cystatin C, a cysteine proteinase inhibitor, inhibits bone resorption in vitro stimulated by parathyroid hormone and parathyroid hormone-related peptide of malignancy. J Bone Miner Res **7:**433–439.
12. Delaisse JM, Boyde A, Maconnachie E, Ali NN, Sear CHJ, Eeckhout Y, Vaes G, Jones SJ 1987 The effects of inhibitors of cysteine proteinases and collagenase on the resorptive activity of isolated osteoclasts. Bone **8:**305–313.
13. Drake FH, Dodds RA, James IE, Connor JR, Debouck C, Richardson S, Lee-Rykaczewski E, Coleman L, Rieman D, Bartlow R, Hastings G, Gowen M 1996 Cathepsin K, but not cathepsins B, L, or S, is abundantly expressed in human osteoclasts. J Biol Chem **271:**12511–12516.
14. Tezuka K-I, Tezuka Y, Maejima A, Sato T, Nemoto K, Kamioka H, Hakeda Y, Kumegawa M 1994 Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. J Biol Chem **269:**1106–1109.
15. Shi GP, Chapman HA, Bhairi SM, DeLeeuw C, Reddy VY, Weiss SJ 1995 Molecular cloning of human cathepsin O, a novel endoproteinase and homologue of rabbit OC2. FEBS Lett **357:**129–134.
16. Brömme D, Okamoto K 1995 Human cathepsin O2, a novel cysteine protease highly expressed in osteoclastomas and ovary molecular cloning, sequencing and tissue distribution. Biol Chem **376:**379–384.
17. Li YP, Alexander M, Wucherpfennig AL, Yelick P, Chen W, Stashenko P 1995 Cloning and complete coding sequence of a novel human cathepsin expressed in giant cells of osteoclastomas. J Bone Miner Res **10:**1197–2202.
18. Inaoka T, Bilbe G, Ishibashi O, Tezuka K-I, Kamegawa M, and Kokubo T 1995 Molecular cloning of human cDNA for cathepsin K: Novel cysteine proteinase predominantly expressed in bone. Biochem Biophys Res Commun **206:**89–96.
19. Fukunishi S, Iwata Y, Mort JS 1997 Immunolocalization of cathepsin K at sites of bone resorption. Trans ORS **22:**586.
20. Bossard MJ, Tomaszek TA, Thompson SK, Amegadzie BY, Hanning CR, Jones C, Kurdyla JT, McNulty DE, Drake FH, Gowen M, Levy MA 1996 Proteolytic activity of human osteoclast cathepsin K: Expression, purification, activation, and substrate identification. J Biol Chem **271:**12517–12524.
21. Brömme D, Okamoto K, Wang BB, Biroc S 1996 Human cathepsin O2, a matrix protein-degrading cysteine protease expressed in osteoclasts. Functional expression of human cathepsin O2 in *Spodoptera frugiperda* and characterization of the enzyme. J Biol Chem **271:**2126–2132.
22. Garnero P, Borel O, Byrjalsen I, Ferreras M, Drake FH, McQueney MS, Foged NT, Delmas PD, Delaisse J-M 1998 The collagenolytic activity of cathepsin K is unique among mammalian proteinases. J Biol Chem **273:**32347–32352.
23. Votta BJ, Levy MA, Badger A, Dodds RA, James IE, Thompson S, Bossard MJ, Carr T, Connor JR, Tomaszek TA, Szewczuk L, Drake FH, Veber DF, Gowen M 1997 Peptide aldehyde inhibitors of cathepsin K inhibit bone resorption both in vitro and in vivo. J Bone Miner Res **12:**1396–1406.
24. Thompson SK, Halbert SM, Bossard MJ, Tomaszek TA, Levy MA, Zhaou B, Smith WW, Abdel-Meguid SS, Janson CA, D'Alessio KJ, McQueney MS, Amegadzie BY, Hanning CR, Desjarlais RL, Briand J, Sarkar SK, Huddleston MJ, James CFI, Carr SA, Garnes KT, Shu A, Heys JR, Bradbeer JN, Zembryki D, Lee-Rykaczewski E, James IE, Lark MW, Drake FH, Gowen M, Gleason JG, Veber DF 1997 Design of potent and selective human cathepsin K inhibitors which span the active site. Proc Natl Acad Sci USA **94:**14249–14254.
25. Yamashita DS, Dodds RA 2000 Cathepsin K, and the design of cathepsin K inhibitors. Curr Pharm Des **6:**1–24.
26. Greenspan A 1991 Sclerosing bone dysplasias—a target-site approach. Skeletal Radiol **20:**561–583.
27. Sarnsethsiri P, Hitt OK, Eyring EJ, Frost HM 1971 Tetracycline-based study of bone dynamics in pycnodysostosis. Clin Orthop **74:**301–312.
28. Cabrejas ML, Fromm GA, Roca JF, Mendez MA, Bur GE, Ferreyra ME, Demarchi C, Schurman L 1976 Pycnodysostosis: Some aspects concerning kinetics of calcium metabolism and bone pathology. Am J Med Sci **271:**215–220.
29. Meredith SC, Simon MA, Laros GS, Jackson MA 1978 Pycnodysostosis. A clinical, pathological, and ultramicroscopic study of a case. J Bone Joint Surg Am **60:**1122–1127.
30. Elmore SM, Nance WE, McGee BJ, Montmollin ME, Engel E 1966 Pycnodysostosis, with a familial chromosome anomaly. Am J Med **40:**273–282.
31. Gelb BD, Shi GP, Chapman HA, Desnick RJ 1996 Pycnodysostosis, a lysosomal disease caused by cathepsin K deficiency. Science **273:**1236–1238.
32. Johnson MR, Polymeropoulos MH, Vos HL, Ortiz de Luna RI, Francomano CA 1996 A nonsense mutation in the cathepsin K gene observed in a family with pycnodysostosis. Genome Res **11:**1050–1055.
33. Saftig P, Hunziger E, Wehmeyer O, Jones S, Boyde A, Rommerskirch W, Moritz J, Schu P, von Figura K 1998 Impaired osteoclastic bone resorption leads to osteopetrosis in cathepsin K-deficient mice. Proc Natl Acad Sci USA **95:**13453–13458.
34. Gowen M, Lazner F, Dodds RA, Kapadia R, Field J, Tavaria M, Bertoncello I, Drake F, Zavarselk S, Tellis I, Hertzog P, Debouck C, Kola I 1999 Cathepsin K knockout mice develop osteoporosis due to a deficit in matrix degradation but not mineralization. J Bone Miner Res **14:**1654–1663.
35. Aibe K, Yazawa H, Abe K, Teramura K, Kumegawa M, Kawashima H, Honda K 1996 Substrate specificity of recombinant osteoclast-specific cathepsin K from rabbits. Biol Pharm Bull **19:**1026–1031.
36. Van Noorden CJ, Vogels IM, Smith RE 1989 Localization and cytophotometric analysis of cathepsin B activity in unfixed and undecalcified cryostat sections of whole rat knee joints. J Histochem Cytochem **37:**617–624.
37. Dodds RA, Connor JR, Drake F, Feild J, Gowen M 1998 Cathepsin K mRNA detection is restricted to osteoclasts during fetal mouse development. J Bone Miner Res **13:**673–682.

38. Dodds RA, Gowen, M and Bradbeer JN 1994 Microcytophotometric analysis of human osteoclast metabolism: The lack of activity in certain oxidative pathways indicates an inability to sustain biosynthesis during resorption. J Histochem Cytochem **42:**599–606.
39. Rieman DJ, McClung HA, Dodds RA, James IE, Holmes S, Drake FH, Gowen M 2000 Biosynthesis and processing of cathepsin K in cultured human osteoclasts (in press).
40. Dodds RA, James IE, Rieman D, Hwang SM, Ahern R, Connor JR, Holmes SD, Thompson S, Veber D, Drake FH, Lark MW, Gowen M 1998 Osteoclast cathepsin K is processed intracellularly prior to osteoclast attachment to bone. Bone **23:**S334.
41. Baron R, Neff L, Louvard D, Courtoy PJ 1985 Cell-mediated extracellular acidification and bone resorption: Evidence for a low pH in resorbing lacunae and localization of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. J Cell Biol **101:**2210–2222.
42. Hart KA, Potts W, Bowyer J, Slynn G, Elston P, Vernon W, Tucker D, Tolley K, Johnstone D 1996 Transgenic mice lacking cathepsin L activity. J Bone Miner Res **11:**S366.
43. Katunuma N 1989 Mechanisms and regulation of lysosomal proteolysis. Rev Biol Cell **20:**35–61.
44. Fallon MD 1984 Biology of the osteoclast. In: Cohn DV, Fujita T, Potts TJ Jr, Talmage RV (eds.) Endocrine Control of Bone and Calcium Metabolism. Elsevier, Amsterdam, The Netherlands, p. 144.
45. Delaisse JM, Vaes G 1992 Mechanisms of mineral solubilization and matrix degradation in osteoclast bone resorption. In: Rifkin BR, Gay CV (eds.) Biology and Physiology of the Osteoclasts. CRC Press, Boca Raton, FL, pp. 289–314.
46. McQueeney MS, Amegadzie BY, D'Alessio K, Hanning CR, McLaughlin MM, McNulty D, Carr SA, Ijames C, Kurdlya J, Jones CS 1997 Autocatalytic activation of human cathepsin K. J Biol Chem **272:**13955–13960.

Address reprint requests to:
Dr. Robert A Dodds
Osiris Therapeutics, Inc.
2001 Aliceanna Street
Baltimore, MD 21231, USA

Received in original form September 24, 1999; in revised form September 8, 2000; accepted October 4, 2000.