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Expression, Maturation, and Rhodamine-Based Fluorescence Assay of Human Cathepsin K Expressed in CHO Cells

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ABSTRACT. Cathepsin K is a cysteine protease that degrades type I human collagen during bone resorption. We have expressed the recombinant human cathepsin K in Chinese hamster ovary (CHO) cells as a pre-proenzyme and demonstrated that it is processed intracellularly to an active enzyme form and that only the proenzyme form is secreted. Immunofluorescence detection of cathepsin K in CHO cells resulted in discrete punctate distribution consistent with a lysosomal localization of the enzyme. With both extract and cell preparations of CHO cells expressing cathepsin K, $[Z\text{-Leu-Arg}]_2$-rhodamine was the best substrate for analyzing cathepsin K activity over background proteases. We have established a cellular-based assay to analyze cell-permeable inhibitors of cathepsin K and validated the assay with detection of intracellular versus extracellular activity, fluorescence-assisted cell sorter (FACS) analysis, and a selective cathepsin K inhibitor. The intracellular activity of cathepsin K was monitored by FACS analysis using the rhodamine substrate, which demonstrated an increased fluorescence over mock-transfected cells that was also inhibitable by (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d). A selective cathepsin K inhibitor, 1,3-bis(CBZ-Leu-NH)-2-propanone, had an IC_{50} of 134 nM in the CHO/Cat K cells, which is the same potency as that measured against a purified enzyme preparation of cathepsin K. Therefore, we have established a system to evaluate intracellular cathepsin K activity and inhibition by cell-permeable inhibitors of this thiol protease. BIOCHEM PHARMACOL **60**;6:759–769, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. cathepsin; cysteine protease; osteoporosis; bone resorption; papain

Osteoporosis has been described as a generalized and progressive loss in bone tissue that results in a weakness in skeletal strength. The major reason for this decreased bone density is believed to be increased bone resorption. Bone resorption is performed primarily by multinuclear giant cells known as osteoclasts. The mechanism by which osteoclasts resorb bone is first through initial attachment to bone and formation of an extracellular compartment that is maintained at a low pH by a proton–ATP pump. This acidic environment then results in the initial demineralization of bone followed by collagen degradation [1, 2]. Collagen constitutes 95% of the organic matrix of bone. Therefore, the proteases involved in collagen degradation are an essential component of bone turnover and in the development of osteoporosis.

Cysteine protease inhibitors such as E64† have been shown to be effective in inhibiting bone resorption [3]. Recently, a cysteine protease from the papain family of proteases, designated cathepsin K, has been cloned and shown to be specifically expressed in osteoclasts [4–8]. Concurrent with the cDNA cloning, an autosomal recessive disorder termed pycnodysostosis was mapped to mutations in the cathepsin K gene, resulting in inactive protein [9, 10]. Since pycnodysostosis is characterized as an osteopetrotic phenotype with a decrease in bone resorption, cathepsin K may be the major cysteine protease involved in osteoclast-mediated bone resorption.

Cathepsin K is synthesized as a 37-kDa pre-proenzyme that is localized to the lysosomal compartment and can autoactivate to the mature 27-kDa enzyme at low pH [11, 12]. Cathepsin K is most closely related to cathepsin S, with 56% sequence identity at the amino acid level. The S_2P_2 substrate specificity of cathepsin K has been shown to be similar to that of cathepsin S, with a preference in the P_1 position for a positively charged residue such as arginine and a hydrophobic residue in P_2 such as phenylalanine or leucine [13, 14]. Cathepsin K has a broad pH activity profile with significant activity between pH 4 and 8,

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† *Abbreviations*: AMC, aminomethylcoumarin; CHO, Chinese hamster ovary; E64, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane; E64c, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane; E64d, (2*S*,3*S*)-*trans*-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester; FACS, fluorescence-assisted cell sorter; FBS, fetal bovine serum; HBSS, Hanks' buffered saline solution; MES, 2-(*N*-morpholino)ethanesulfonic acid; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SBTI, soybean trypsin inhibitor; and Z, benzyloxycarbonyl.

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allowing for good catalytic activity in the resorption lacunae of osteoclasts, where the pH is 4–5. Human type I collagen is the major form of collagen in bone and is a good substrate for cathepsin K [15]. Antisense oligonucleotide experiments for cathepsin K have demonstrated partial efficacy in preventing bone resorption in an *in vitro* setting [16]. The crystal structure of cathepsin K has been solved [17, 18], and selective peptide-based inhibitors of cathepsin K have been developed [14, 19]. Analyses of these inhibitors should provide further evidence for the role of cathepsin K in bone resorption and in pathological disorders such as osteoporosis.

The major *in vitro* model of bone resorption is a pit formation assay in which purified osteoclasts are cultured on bone slices with no specific readout for cathepsin K activity. Since osteoclasts are believed to create tight junctions with the bone matrix and degraded collagen is transcytosed through osteoclasts [20, 21], cell-permeable inhibitors would be required to obtain therapeutic benefit. Therefore, a cell-based assay was required for the assessment of intracellular inhibition by cathepsin K inhibitors. A stable cell line expressing cathepsin K was isolated, and, using dipeptide rhodamine substrates, we established a whole cell assay for the measurement of cathepsin K activity.

MATERIALS AND METHODS

Materials

Oligonucleotide primers were synthesized at Research Genetics. Prestained molecular weight markers were purchased from Life Technologies. Fluorogenic peptides were purchased from Bachem, Molecular Probes, or Novabiochem. The $[Z\text{-Leu-Arg}]_2$-rhodamine substrate was custom synthesized at Anaspec. Cysteine protease inhibitors E64, E64c, and E64d were purchased from the Sigma Chemical Co. Purified cathepsin K [17] and 1,3-bis(CBZ-Leu-NH)-2-propanone were obtained from Axys Pharmaceuticals.

Cloning and Sequencing of the Cathepsin K cDNA

The full-length cDNA of human cathepsin K [4, 7] was amplified by PCR from a commercially available human bone marrow cDNA library (Clontech) using the following oligonucleotides as primers for the PCR: 5′-ACAGATTTCCATCAGCAG-3′ and 5′-AGTAGGAAGGATCATTTG-3′. The PCR was performed in a GeneAmp 2400 PCR system from Perkin-Elmer for 30 cycles of denaturation (94°, 30 sec), annealing (55°, 30 sec), and extension (72°, 1 min) using the Expand High Fidelity PCR System (Boehringer Mannheim). Following this primary amplification, a second PCR was performed using two specific nested primers containing *Eco*RI and *Not*I restriction sites: 5′-GAAGCCAGACGAATTCACAGATTTCCATCAGCAG-3′ and 5′-ATTAGTCTTGCGGCCGCGGATCCTCACATCTTGGGGAAGCTGGCCAGGTT-3′. The resulting PCR product then was digested and ligated into an *Eco*RI–*Not*I pcDNA3.1 vector (Invitrogen). Sequence analysis was performed on an ABI 373A DNA sequencer (Applied Biosystems) using the Dye Deoxy Terminator Kit (Applied Biosystems), and the cloned cDNA was found to be identical to the published sequence [4].

Construction of a Cathepsin K Stable Cell Line

Stable expression of the vector only or the pcDNA3.1/hCat K construct was achieved by performing a liposome-mediated transfection into CHO cells (CHO-K1; American Type Culture Collection CCL-61) using the Lipofectamine reagent (Life Technologies) as described in the supplier's instructions, followed by clonal selection of stable transfectants. Cells were grown as monolayer cultures in HyQ-CCM5 medium (HyClone), supplemented with 2% heat-inactivated FBS, 100 μg/mL of streptomycin, 100 U/mL of penicillin, 100 μg/mL of gentamicin, and 500 μg/mL of G418 (Life Technologies) for selection. Vector-only-expressing cell clones (mock) were identified by Southern blot analysis (data not shown), and stable human cathepsin K-expressing cell clones were identified by western blot analysis and determination of enzyme activity as described below.

Immunodetection of Cathepsin K

Cell pellets were resuspended in PBS and were sonicated for 3 × 10 sec using a Kontes Ultrasonic Disrupter. The suspension was centrifuged at 1000 *g* for 10 min at 4°, and the resulting supernatant was utilized as the cellular extract for determination of cathepsin K expression and activity. Protein concentrations were determined using the Pierce Coomassie Protein Reagent (Pierce) as described in the manufacturer's instructions. For immunoblot analysis, cellular extracts were prepared as described above with the exception that they were prepared in PBS supplemented with the Complete Protease Inhibitor Cocktail (Boehringer Mannheim) at 2-fold the suggested concentration in the manufacturer's instructions. The serum-free cell media were concentrated using Centriplus-10 concentrators (Amicon) according to the manufacturer's instructions. Protein samples were resolved by SDS–PAGE on precast 4–20% Tris–glycine acrylamide gels (Novex) and electrophoretically transferred to PVDF membranes using a Novex immunoblot transfer apparatus, according to the manufacturer's instructions. Nonspecific sites were blocked with 5% non-fat dry milk in PBST (PBS, 0.05% Tween) for 1 hr at room temperature, and the membranes were washed twice in PBST, for 5 min each. Blots then were incubated for 1 hr with a 1/20,000 dilution in 1% BSA/PBST of an anti-human Cat K rabbit polyclonal antibody (Axys Pharmaceuticals) or an anti-human Cat S polyclonal antibody (produced by Research Genetics against the antigenic residues 208–222 of cathepsin S) and washed four times in PBST, for 15 min. Finally, blots were incubated for 1 hr with a 1/3000 dilution in 1% BSA/PBST of a horseradish

peroxidase-linked anti-rabbit IgG antibody (Amersham), and washed again four times in PBST. Immunoblot analysis was performed using Renaissance Western Blot Chemiluminescence Reagent (NEN) according to the manufacturer's instructions.

Immunofluorescence Localization of Cathepsins

Stable CHO/mock or CHO/hCat K stable cell lines were trypsinized and seeded at 500,000 cells/well in sterile 35-mm tissue culture dishes containing sterile coverslips. After an overnight incubation at 37°, cells were washed in PBS and incubated for 15 min at −20° in a fresh methanol:1% formaldehyde solution. After another 10 min of incubation at room temperature, cells were washed again and placed briefly in PBS containing 50 mM NH_4Cl. Then cells were rinsed again in PBS and incubated for 20 min in 2% BSA and 0.2% gelatin in PBS. After washing in PBS, cells were incubated in a 1/1000 dilution of anti-human Cat K mouse monoclonal antibody (Medicorp) or anti-human Cat B sheep polyclonal antibody (Serotec). After a 90-min incubation, cells were washed in PBS and incubated for 45 min in a 1/200 dilution of the fluorescein isothiocyanate (FITC)-conjugated secondary antibody: donkey anti-mouse IgG (Amersham). Finally, cells were washed in PBS, and the coverslips were inverted on a slide containing a drop of the Prolong Anti-fade Kit reagent (Molecular Probes). The slides were observed using a Zeiss Axiophot fluorescence microscope, and the exposure of the CHO/mock cells incubated with anti-cathepsin K was increased to visualize the cellular background level of fluorescence.

Enzyme Assays

The enzymatic activity of purified cathepsin K (3 nM) and cellular extracts (5 μg/mL of protein) was determined from the rate of hydrolysis of the $[Z\text{-Leu-Arg}]_2$-rhodamine or AMC substrate. Assays were performed at room temperature in 200 μL of 50 mM MES buffer, pH 5.5, containing 2.5 mM EDTA, 2.5 mM dithiothreitol, 10% DMSO (buffer A). Substrate hydrolysis was monitored over a period of 10 min at room temperature in a Cytofluor 4000 fluorescent plate reader (Perseptive Biosystems). All activity measurements were calculated as initial rates over the first 5 min of the reaction.

Cathepsin K activity in intact cell assays was determined by incubation of cells with 5 μM $[Z\text{-Leu-Arg}]_2$-rhodamine substrate, unless otherwise indicated, in the presence of cystatin. Twenty-four hours prior to the experiment, mock or Cat K-expressing stable cell lines were trypsinized and dispensed into sterile 96-well cell culture plates (Nunc) at 50,000 cells per well. The next day, cells were washed twice in Ca^{2+}- and Mg^{2+}-containing PBS using the Skanwasher 300 plate washer (Skatron), and 200 μL HBSS, pH 7.4, supplemented with 15 mM HEPES, 20 μg/mL of cystatin (Calbiochem), and 100 μg/mL of soybean trypsin inhibitor (Sigma) was added to every well. After a 15-min preincubation at room temperature, cells were challenged by the addition of 2 μL of a 500 μM stock solution of $[Z\text{-Leu-Arg}]_2$-rhodamine substrate (5 μM final concentration), unless otherwise indicated, dissolved in DMSO. Substrate hydrolysis was monitored at room temperature in a Cytofluor 4000 fluorescent plate reader (Perseptive Biosystems). Inhibition of cathepsin activity in enzyme suspensions or whole cells was performed by preincubation with 1 μL of vehicle or inhibitors for 15 min at room temperature prior to the initiation of the reaction with $[Z\text{-Leu-Arg}]_2$-rhodamine. All inhibitors were prepared as stock solutions in DMSO, and the assays were performed with a final DMSO concentration of 1.5%.

Flow Cytometry Analysis

Stable CHO/mock or CHO/hCat K stable cell lines were trypsinized and resuspended in Ca^{2+}- and Mg^{2+}-free PBS containing 20 μg/mL of cystatin and 100 μg/mL of soybean trypsin inhibitor at a density of 1 million cells/mL. A 15-min preincubation at 37° in the presence of vehicle or inhibitors was performed prior to the addition of 5 μM $[Z\text{-Leu-Arg}]_2$-rhodamine substrate. After a 15-min incubation at 37°, flow cytometry analysis was performed using a FACSCalibur system (Becton Dickinson) equipped with CellQuest software. Voltage and fluorescence compensation adjustments were performed before each experiment using CaliBRITE beads (Becton Dickinson) and FACSComp software (Becton Dickinson). A minimum of 10,000 events were collected for each sample, and cells were gated to exclude cell debris and abnormally large or aggregated cells from analysis.

RESULTS

Expression of Human Cathepsin K in CHO Cells

A full-length cDNA encoding the signal sequence, prodomain, and mature enzyme of human cathepsin K was PCR cloned from a bone marrow cDNA library. The cathepsin K cDNA was subcloned into pcDNA3.1 and transfected into CHO cells. Stable cell lines containing pcDNA3.1 vector with and without (mock) the cathepsin K cDNA insert were obtained by selection with the neomycin analogue G418 for 1 month by limited dilution cloning. Extracellular and intracellular cathepsin K expression was determined by immunoblot analysis. The media and cellular extracts from the CHO/mock and CHO/Cat K stable cell lines were prepared, subjected to SDS–PAGE, and analyzed by immunoblot using a polyclonal antiserum to cathepsin K (provided by Axys Pharmaceuticals). The medium of the mock cells contained a cross-reacting band that did not correlate with a relevant molecular mass for cathepsin K (Fig. 1). The extract of the CHO/mock cells contained no protein signal detectable by immunoblot with the cathepsin K antiserum. The medium of the CHO/Cat K stable cell line contained an immunoreactive protein of 37 kDa, which co-migrated with a standard of pro-cathepsin K.

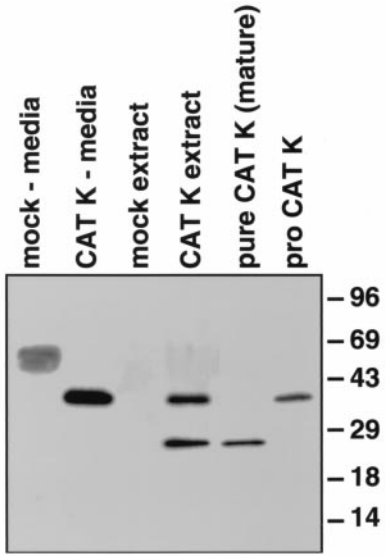

FIG. 1. Western blot analysis of cathepsin K in stable mock and cathepsin K-expressing CHO cells. The serum-free media of the stable mock and cathepsin K-expressing cells were collected and concentrated. Cells were pelleted, resuspended in PBS, sonicated, and centrifuged at 1000 *g* for 10 min. Then, protein samples (3 μg) from the media and the 1000 *g* supernatants were separated by electrophoresis, transferred to a PVDF membrane, and probed with a human cathepsin K specific antiserum. Human pro-mature and mature cathepsin K standards were included (10 ng), and molecular weight markers are designated.

An immunoblot of the cellular extract (1000 *g* supernatant) prepared from the CHO/Cat K cells contained two proteins migrating at 27 and 37 kDa, corresponding to mature and pro-cathepsin K, respectively. This suggests that intracellular activation to the mature form occurs in CHO cells. Approximately 50% of the expressed cathepsin K enzyme was in the mature form. This intracellular activation of cathepsin K to the mature form was not restricted to CHO cells, since a transient transfection in COS-7 cells resulted in a similar ratio of mature to pro-cathepsin K (data not shown). About 40% of the total cathepsin K expressed by CHO cells was secreted into the media. Analysis of cathepsin S expression was also performed in CHO cells, and no detectable immunoreactive band corresponding to cathepsin S was measurable (data not shown).

Immunofluorescence Localization of Cathepsin K and B Expressed in CHO Cells

Cathepsin K has been localized previously to the lysosomal compartment within osteoclasts [12]. We performed immunofluorescence analysis on the CHO/mock and CHO/Cat K cells to localize the expressed protein (Fig. 2). The cathepsin K monoclonal antibody detected a faint signal of fluorescence in the CHO/mock cells, suggesting little or no endogenous cathepsin K in these cells and consistent with the lack of protein detectable by immunoblot analysis. The CHO/Cat K cells stained with the cathepsin K monoclonal antibody resulted in an intense punctate staining throughout the cytoplasm. This intense punctate signal for cathepsin K is consistent with a distribution of the immunoreactive protein in the lysosomal compartment. Cathepsin B also was detected in CHO cells by immunostaining with a polyclonal antibody and was found to be diffusely present throughout the cell, excluding the nucleus (data not shown).

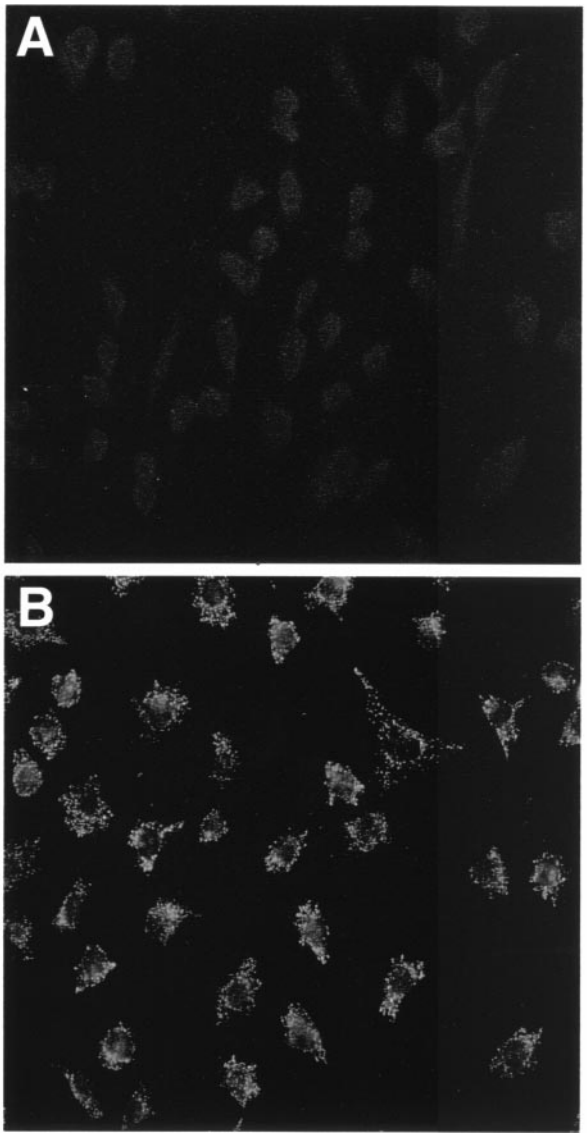

FIG. 2. Immunofluorescence localization of cathepsin K in stable mock and cathepsin K-expressing CHO cells. Stable mock and cathepsin K-expressing cells were fixed and permeabilized in a fresh methanol:1% formaldehyde solution for 15 min at −20°, and then stained with the indicated primary antibody and the corresponding FITC-conjugated secondary antibody. (A) Stable CHO/mock cells and (B) stable CHO/Cat K cells were stained with a cathepsin K monoclonal antibody. Exposure of panel A was increased manually in comparison with exposures of panel B in order to detect the background level of fluorescence.

Enzymatic Activity of Cathepsin K in Cellular Extracts

Cathepsin K is a cysteine protease with a substrate S_2P_2 specificity of a positively charged residue such as arginine in

P_1 and a large hydrophobic residue in P_2. We compared the utilization of various dipeptide substrates for recombinant cathepsin K expressed in CHO cells. Two fluorophores, AMC and rhodamine, were used for the peptide substrates in the P′ position. Cellular extracts of CHO/mock and CHO/Cat K cells were assayed for the ability to hydrolyze the various substrates at a concentration of 5 μM, and the results are depicted in Fig. 3. The data show that Z-Leu-Arg with either AMC or rhodamine conjugates provided the largest measurable activity among the tested substrates. The activity of the cathepsin K extract was at least 12-fold higher than that of the mock extract with Z-Leu-Arg substrates, demonstrating that the expressed cathepsin K has significant activity above endogenous proteases in CHO cells. The use of Z-Phe-Arg substrates resulted in 1.8- to 3-fold lower activity than the corresponding Z-Leu-Arg substrates (Fig. 3). The most selective cathepsin K substrate, Z-Pro-Arg,* gave no detectable activity in the mock extract with either the AMC or rhodamine conjugate, but the level of activity in the CHO/Cat K extract appeared too low for use in development of a cellular-based assay. The amount of immunoreactive cathepsin K was quantitated by comparison to a purified cathepsin K standard, and the mature CHO cathepsin K was estimated to be 15% as active as the purified protein, using the Z-Leu-Arg-AMC substrate. Several assays also resulted in negative fluorescence, and from our experience, this is suggestive of substrate precipitation or micelle formation. The rhodamine conjugates were tested along with AMC, since rhodamine dipeptides have been described as more cell-permeable substrates [22].

Cystatins are a family of endogenous protein inhibitors of cathepsins. To confirm that the activity measured for the dipeptide substrates described was due to a cathepsin, activity in the presence or absence of cystatin was measured. The CHO/Cat K extract or purified cathepsin K was preincubated for 15 min with various concentrations of cystatin and assayed with the Z-Leu-Arg-Rho substrate (Fig. 4). The IC_{50} for inhibition of activity of the purified cathepsin K by cystatin was 200 ng/mL, and 90 ng/mL for the CHO/Cat K extract. This demonstrated that the expressed cathepsin K in CHO cells is inhibitable in a similar fashion as is the purified enzyme.

Whole Cell Assay for Cathepsin K

Since cathepsins have been shown to be secreted and active extracellularly, several protease inhibitors were tested with CHO/Cat K cells to eliminate interference by measurement of extracellular protease activities. To analyze intracellular cathepsin K activity, CHO cells containing the mock vector or expressing cathepsin K were seeded in 96-well tissue culture plates as described in Materials and Methods, and the activity was determined using the Z-Leu-Arg-Rho substrate. The assay with cells (Fig. 5) was performed in the

* Percival MD, personal communication. Cited with permission.

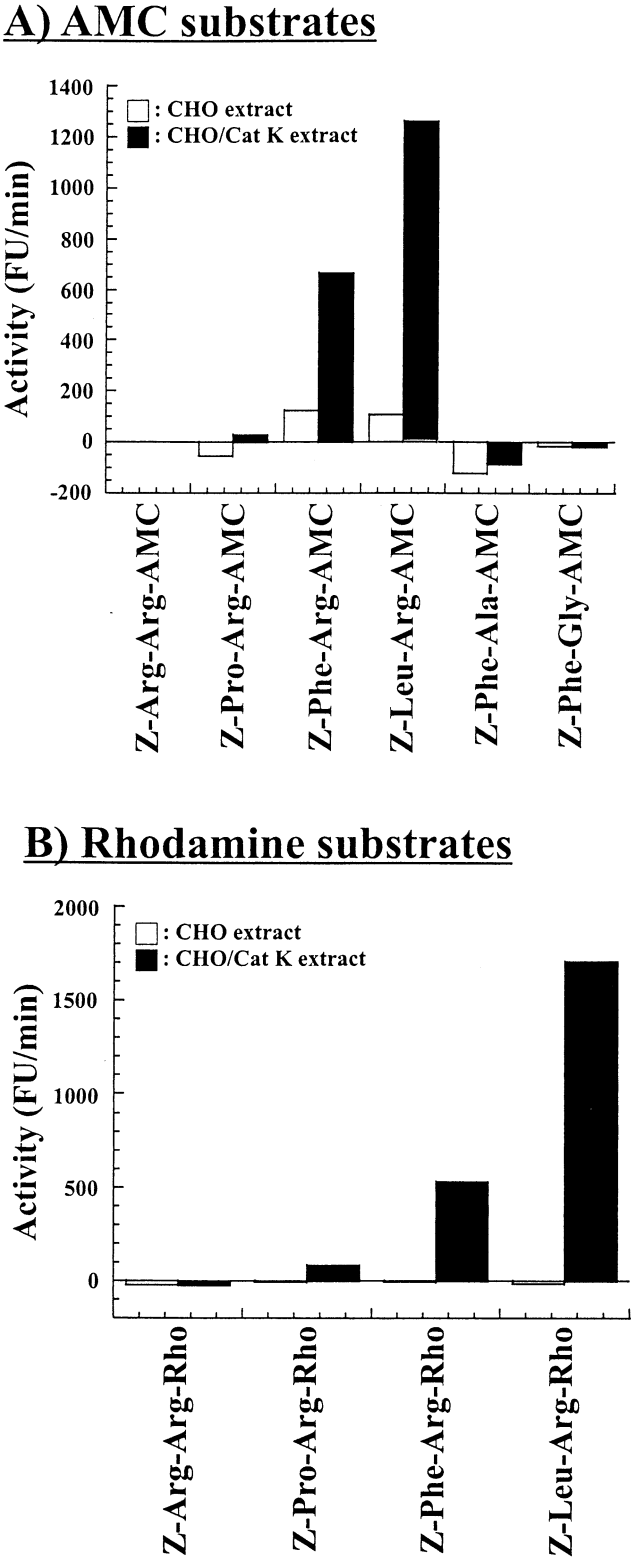

FIG. 3. Hydrolysis of fluorogenic dipeptide substrates by extracts of stable mock and cathepsin K-expressing CHO cells. Stable mock and cathepsin K-expressing cells were pelleted, and 1000 g supernatants were prepared and assayed at 5 μg/mL in buffer A by measurement of the hydrolysis of fluorogenic peptide substrate at a fixed concentration of 5 μM. The reported enzymatic activities constitute the initial rate of substrate hydrolysis, and each data point is an average of duplicates. FU = fluorescence units.

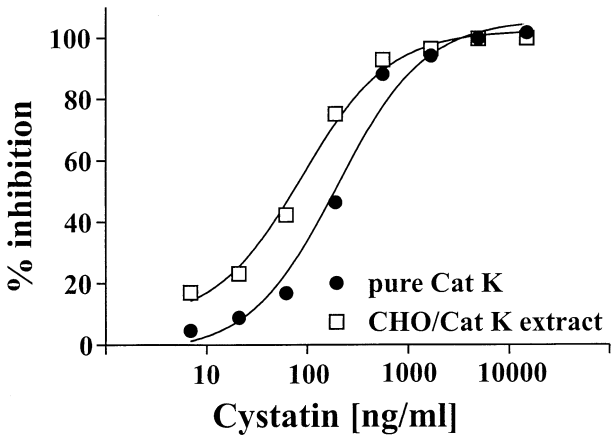

FIG. 4. Inhibition of cathepsin K activity by cystatin. Supernatants (1000 g) from stable mock and cathepsin K-expressing cells (5 μg/mL) and the pure cathepsin K enzyme (3 nM) were resuspended in buffer A and assayed for substrate hydrolysis in the presence of variable concentrations of cystatin. The enzyme suspensions were preincubated with the cysteine protease inhibitor for 15 min prior to the initiation of the reaction with 5 μM Z-Leu-Arg-Rho as substrate. Each data point is an average of duplicates and is reported as a percentage of inhibition of the control reaction (pure Cat K control: 3534 ± 321 FU/min; CHO/Cat K control: 2195 ± 81 FU/min).

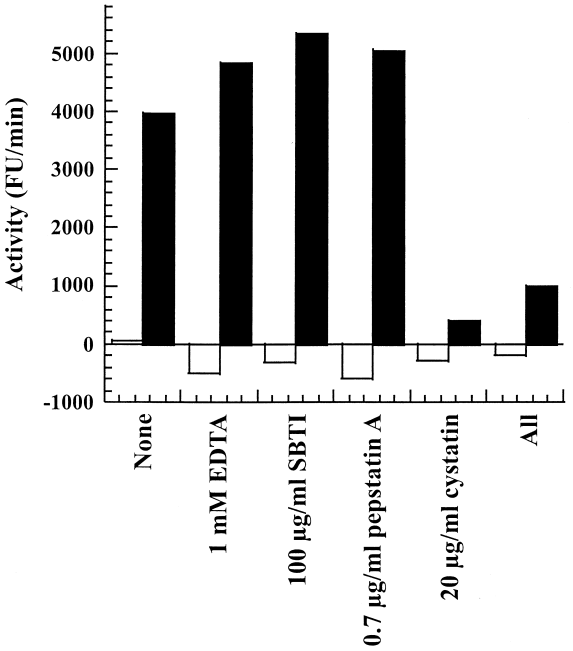

FIG. 5. Effect of different protease inhibitors on the hydrolysis of Z-Leu-Arg-Rho by mock or CHO/Cat K cells. Twenty-four hours prior to the experiment, stable mock (□) or human cathepsin K-expressing (■) cells were seeded in sterile 96-well cell culture plates. The next day, cells were washed twice with PBS, and then HBSS, pH 7.4, containing 15 mM HEPES was added to every well. Cells then were assayed for substrate hydrolysis in the presence of the designated protease inhibitors. The cells were preincubated with the inhibitors for 15 min prior to the initiation of the reaction with 5 μM Z-Leu-Arg-Rho as substrate. The reported enzymatic activities constitute the initial rate of substrate hydrolysis, and each data point is an average of duplicates.

presence of the metalloprotease inhibitor (1 mM EDTA), the trypsin inhibitor (SBTI), the aspartyl protease inhibitor (pepstatin A), and the cysteine protease inhibitor (cystatin). The only inhibitor that resulted in a decrease in activity in the CHO/Cat K cells was cystatin, whereas the other protease inhibitors had little effect on the activity. Since the concentration of cystatin used completely inhibits the activity of both purified and CHO extracts containing cathepsin K activity, it was utilized to inhibit extracellular cysteine protease activity. Cystatin is a 12-kDa reversible inhibitor of cysteine proteases of the cathepsin family that does not permeate into cells (cell permeability was tested in CACO-2 cells; Guay J and Mancini J, unpublished results). The mock cells (Fig. 5) contained no measurable activity in the absence or presence of any of the protease inhibitors. One plausible explanation for the increased extracellular activity in the cathepsin K-expressing cells as compared with the mock cells is that the overexpressed cathepsin K may be activated or may activate several proteases that are secreted. Cystatin and SBTI were utilized in all whole cell assays, and the SBTI was maintained, since cells were subcultured with the use of trypsin.

We have described above the use of dipeptide substrates for the optimal measurement of cathepsin K activity in the extracts, and we also repeated the experiments with whole cells in the presence of cystatin and SBTI (Fig. 6). The results obtained in the whole cells were comparable to those obtained for the extract, in which the Z-Leu-Arg peptides conjugated with AMC or rhodamine are very good substrates to measure intracellular activity. The major difference as compared with the cathepsin K extract preparation was that Z-Pro-Arg-Rho, Z-Phe-Arg-Rho, and Z-Leu-Arg-Rho were all good substrates for CHO/Cat K cells, whereas Z-Leu-Arg was the best rhodamine-conjugated peptide substrate in the extract. A possible explanation for this discrepancy is that the measurement of intracellular activity is dependent not only on the substrate but also on its permeability into the cell. The Z-Leu-Arg-Rho substrate was preferred over Z-Pro-Arg-Rho, since a 50% increase of activity was achieved with Z-Leu-Arg-Rho as compared with Z-Pro-Arg-Rho. Also, Z-Phe-Ala-AMC was a good substrate for both the CHO/mock and CHO/Cat K cells, demonstrating an endogenous protease activity in the CHO cells.

Extracellular proteases accounted for 90% of the measurable protease activity in cell-based assays with the dipeptide substrate Z-Leu-Arg-Rho (Fig. 5). This was attenuated with cystatin, but the question still arises whether the residual activity in the presence of cystatin is truly intracellular or due to an incomplete inhibition of extracellular activity by cystatin. To address this question, several approaches were investigated. The first approach is detailed in Fig. 7, in which the reaction was initiated with Z-Leu-Arg-Rho, and after 3 min the media were removed from the cells. These cells then were replenished with fresh media without any further substrate addition, and the activity of the cells and

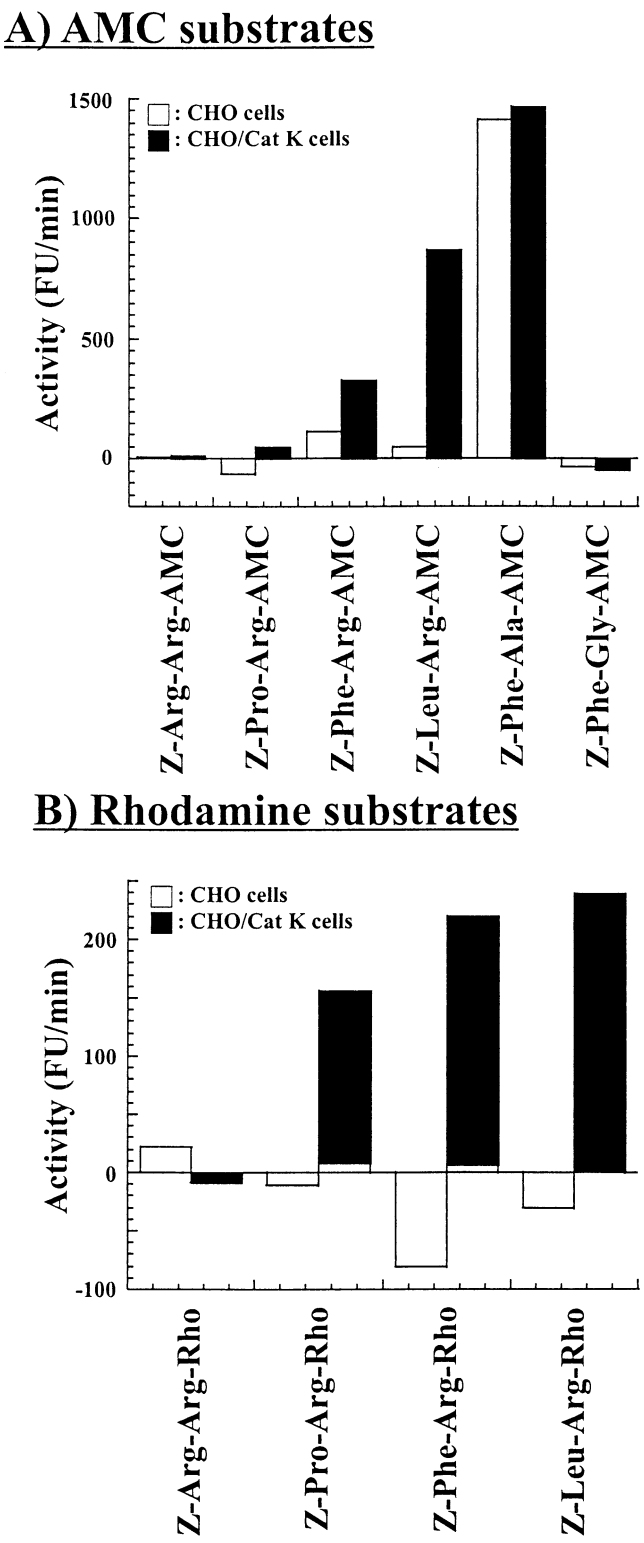

FIG. 6. Activity in CHO/Cat K cells versus extracellular medium. Stable human cathepsin K-expressing cells in HBSS, pH 7.4, supplemented with 15 mM HEPES, 20 μg/mL of cystatin, and 100 μg/mL of SBTI were incubated with 5 μM Z-Leu-Arg-Rho, and its hydrolysis was monitored at room temperature at regular intervals. After 3 min, the media were removed, and the cells were replenished with fresh substrate-free media. Again, the substrate hydrolysis of the replenished cells and the removed media was followed by fluorescence monitoring (representative of N = 2 experiments).

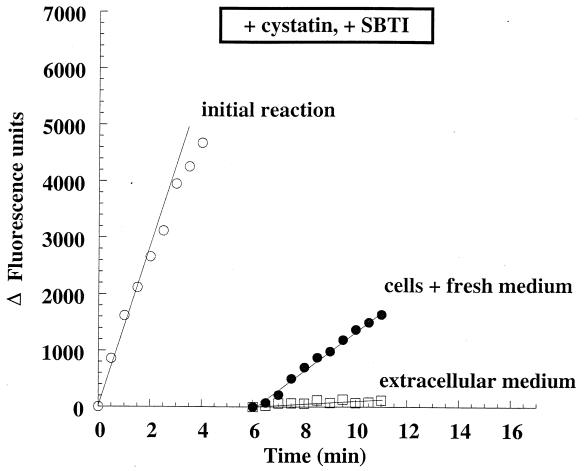

FIG. 7. Hydrolysis of fluorogenic dipeptide substrates by stable mock and cathepsin K-expressing CHO cells. Stable mock or human cathepsin K-expressing cells were seeded in sterile 96-well cell culture plates. The next day, cells were washed twice with PBS, and then HBSS, pH 7.4, supplemented with 15 mM HEPES, 20 μg/mL of cystatin, and 100 μg/mL of SBTI was added to every well. After 15 min of preincubation, the reactions were initiated by the addition of 5 μM concentrations of the designated peptide substrates. The activities constitute the initial rate of substrate hydrolysis, and each data point is an average of duplicates.

of the removed media was followed by fluorescence monitoring. As seen in Fig. 7, the cells containing fresh media continued to increase in fluorescence, demonstrating cleavage of the originally added peptide substrate, whereas the removed media contained no detectable activity. This experiment demonstrated that in the presence of extracellular protease inhibitors, the cells rather than extracellular proteases are responsible for the hydrolysis of the Z-Leu-Arg-Rho substrate. Also, removal of the media in the absence of extracellular protease inhibitors resulted in detectable cleavage of the fluorogenic substrate, demonstrating that the substrate concentration was not limiting, and that this activity was inhibited completely upon the addition of cystatin and SBTI (data not shown).

A second approach to evaluate the contribution of cathepsin K activity is with the use of cysteine protease inhibitors including a selective cathepsin K inhibitor, 1,3-bis(CBZ-Leu-NH)-2-propanone. E64 is a cysteine protease inhibitor, and our results show that it was 19-fold more potent at inhibiting purified Cat K than the whole cell activity (Fig. 8A). E64c, an analogue of E64, was only 8-fold less potent in the CHO/Cat K cell assay as compared with the pure enzyme and CHO/Cat K extract (Fig. 8B) [23]. The selective cathepsin K inhibitor 1,3-bis(CBZ-Leu-NH)-2-propanone, which was reported to have a $K_{i,app}$ of 22 nM for cathepsin K, 0.34 μM for cathepsin L, 1.3 μM for cathepsin B, and 0.89 μM for cathepsin S, was used to confirm that the activity measured in the whole cell assay was due to cathepsin K [24]. 1,3-Bis(CBZ-Leu-NH)-2-propanone was tested in the CHO/Cat K cells in an assay similar to that performed with E64 (see Fig. 8C), and this

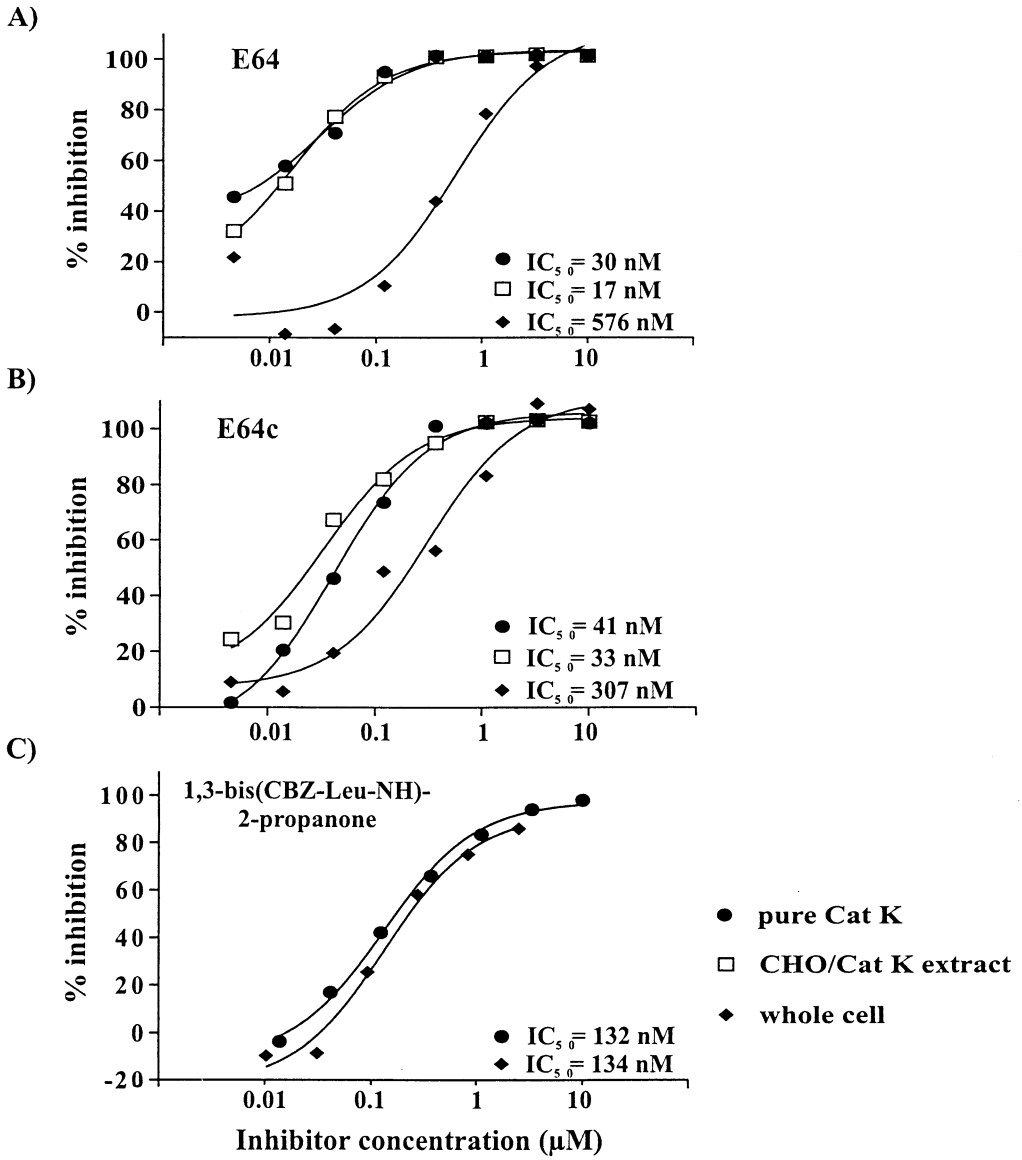

FIG. 8. Inhibition of pure cathepsin K and CHO/Cat K activity by E64 (A), E64c (B), and 1,3-bis(CBZ-Leu-NH)-2-propanone (C). Stable mock or human cathepsin K-expressing cells were resuspended in HBSS, pH 7.4, supplemented with 15 mM HEPES, 20 μg/mL of cystatin, and 100 μg/mL of SBTI. Extracts of stable mock or cathepsin K-expressing cells and pure cathepsin K enzyme were resuspended in buffer A. Cells and enzyme suspensions were preincubated with the designated inhibitors for 15 min prior to the initiation of the reaction with 5 μM Z-Leu-Arg-Rho as substrate (values represent the average of duplicates).

selective cathepsin K inhibitor had an IC_{50} of 134 nM in the cell-based assay, which is comparable to the IC_{50} of 132 nM against the pure cathepsin K enzyme. The IC_{50} in the CHO/Cat K cells was approximately 6-fold higher than the $K_{i,app}$ previously published and at least 2.6-fold lower than the IC_{50} for cathepsin B, L, or S. Therefore, the activity measured in the CHO/Cat K cells was due primarily to cathepsin K activity, based on the data obtained with a selective cathepsin K inhibitor.

The inhibitor data provided further evidence that the assay with the rhodamine substrate in the presence of cystatin measures intracellular cathepsin K activity. A third approach to validate the cell-based assay for cathepsin K was the use of a fluorescence-assisted cell sorter (FACS analysis). FACS analysis of the CHO/Cat K cells in the presence or absence of the substrate Z-Leu-Arg-Rho (Fig. 9A) demonstrated an increase in the fluorescence of cells in the presence of the substrate. Furthermore, the fluorescence intensity of CHO/Cat K cells in the presence of Z-Leu-Arg-Rho was 2-fold higher than that of the mock cells (Fig. 9B). This increase in fluorescence of the CHO/Cat K cells was inhibitable by E64d, the ester of E64c (Fig. 9C), confirming the use of this assay for the measurement of intracellular cathepsin K activity.

DISCUSSION

Cathepsin K has been shown to be selectively expressed in osteoclasts and to play an important role in bone resorption. The modulation of this activity by a selective inhibitor may provide a useful therapeutic approach for the

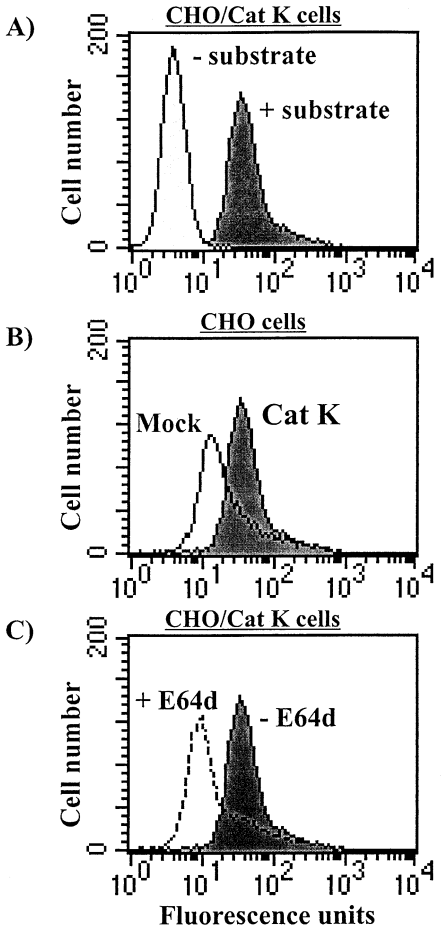

FIG. 9. Flow cytometry analysis of stable mock and cathepsin K-expressing CHO cells. Stable mock or human cathepsin K-expressing cells were resuspended in Ca^{2+}- and Mg^{2+}-free PBS containing 20 μg/mL of cystatin and 100 μg/mL of SBTI. They were preincubated with inhibitors for 15 min at 37° prior to the addition of 5 μM Z-Leu-Arg-Rho as substrate. Flow cytometry analysis was performed after a 15-min incubation with the substrate at 37°: (A) CHO/Cat K cells in the absence and presence of substrate; (B) mock-transfected and CHO/Cat K cells; and (C) CHO/Cat K cells with the substrate in the absence or presence of 10 μM E64d.

treatment of osteoporosis. We have developed a cell-based assay for cathepsin K that can be used as a measure of intracellular activity and in the identification of cell-permeable inhibitors. The expression of the pre-pro-mature enzyme and its activation to the mature enzyme (50%) within the cell is consistent with the autoactivation of cathepsin K. Purified pro-cathepsin K has been shown to be activated to the mature form by lowering the pH to 4.0 [25]. This decrease in pH is believed to cause a change in the conformation of the protein, which then allows the active site cysteine to autocatalyze the activation to the mature form of the enzyme. As seen by using a CHO expression system and immunofluorescence staining, cathepsin K had a localization that was consistent with the lysosomal compartment. Since lysosomes can have pH values of 4–5, cathepsin K could be activated autocatalytically within the lysosome. We also detected cathepsin K secreted into the media of the CHO cells, and this is consistent with reports of secretion of cysteine proteases of the papain family [26]. It is interesting that the secreted form of the enzyme has a molecular weight consistent with the pro-mature cathepsin K and is not processed. This may be due to several factors, one of which is that the medium of these cells has a neutral pH environment that is not conducive to activation. All of the mature form of cathepsin K detected in the CHO cells was retained within the cells. We have confirmed that this intracellular activation was not a result of protease activation during cell lysis, since performing the lysis in the presence or absence of a cocktail of protease inhibitors (containing serine, cysteine, metallo-, and aspartyl protease inhibitors) resulted in the same activation of cathepsin K. In an environment of bone resorption, cathepsin K may be secreted as the pro-mature form, but then has to be activated to the mature form in the surrounding milieu of the osteoclasts. The autocatalytic activation may proceed due to the formation of resorption lacunae (formed by osteoclasts), which have an acidic microenvironment due to the proton pump that functions in osteoclasts to demineralize bone.

Cathepsin L and S are the closest homologues to cathepsin K, with amino acid sequence identities of 51 and 56%, respectively. Cathepsin K also has a very similar S_2S_1 substrate specificity to cathepsin S, which would create difficulty in establishing a recombinant cell-based assay specific for cathepsin K. The increased activity measured in CHO/Cat K cells as compared with mock-transfected cells, using the peptide substrates, demonstrated that cathepsin K was expressed to a sufficient level to detect activity above endogenous proteases, and no significant level of cathepsin S was detected in the CHO cells. Cathepsin B is a ubiquitously expressed protease and was detected in the CHO cells (data not shown). This did not pose a problem for assay development, since the cathepsin B substrate Z-Arg-Arg-AMC [27] was not utilized efficiently by the CHO cells. Two other peptide substrates, Z-Phe-Ala-AMC and Z-Phe-Gly-AMC, served as sufficient negative controls in the cellular extracts, since they are not valid substrates for cathepsin K, L, S, or B.

A significant amount of extracellular proteolytic activity was detectable and could be inhibited by cystatin. Several exhaustive methods were used to establish that the activity measured in the CHO/Cat K stable cell line was intracellular. In the presence of the cell-impermeable inhibitors cystatin and SBTI, the extracellular media removed from cells after the initiation of the reaction with Z-Leu-Arg-Rho contained no detectable activity. The cells remaining after washing continued to exhibit an increase in fluorescence, showing that the cells were responsible for the observed proteolytic activity and that the substrate was indeed cell-permeable. Also, the FACS experiments demonstrated that CHO/Cat K cells had an increase in fluorescence as compared with CHO/mock cells in the presence of the substrate Z-Leu-Arg-Rho. This increase in fluorescence was also inhibitable by E64d. Finally, a selective cathepsin K inhibitor was shown to have an IC_{50} in the CHO/Cat K

cells within 6-fold of the $K_{i,app}$ of the compound for the purified cathepsin K enzyme and with an IC_{50} similar to that of our pure cathepsin K tested under similar substrate concentrations. All combined, the experimental evidence provides substantial support for the development of an assay to detect intracellular cathepsin K activity. Since cathepsin K is a protease that can cleave large protein substrates such as collagen, one possibility that we cannot exclude is that cathepsin K may be activating another protease, which then can cleave the fluorescent rhodamine or AMC peptide substrate. Although this is possible, the cellular assay is still valid in detecting cathepsin K activity either directly or indirectly, and this is confirmed with the use of a selective cathepsin K inhibitor [1,3-bis(CBZ-Leu-NH)-2-propanone].

Recently, a mouse knockout of cathepsin K has been developed, which results in an increase in trabecular bone and an overall decrease in bone resorption similar to the human gene mutation linked to pycnodysostosis [28]. These reports reinforce the importance of cathepsin K in pathophysiological conditions such as osteoporosis, and provide increased impetus for the development of cathepsin K inhibitors. Considering the limited availability of osteoclasts and the lack of established cell lines, this assay will provide a useful tool for the rapid analysis and development of cell-permeable cathepsin K inhibitors. Also, we have demonstrated conclusively that recombinant cathepsin K is expressed and activated to the mature form of the enzyme intracellularly and the secreted form of the enzyme is the pro-cathepsin K.

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