Proteolytic Excision of a Repressive Loop Domain in Tartrate-resistant Acid Phosphatase by Cathepsin K in Osteoclasts*

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Tartrate-resistant acid phosphatase (TRAP) is a metallophosphoesterase participating in osteoclast-mediated bone turnover. Activation of TRAP is associated with the redox state of the di-iron metal center as well as with limited proteolytic cleavage in an exposed loop domain. The cysteine proteinases cathepsin B, L, K, and S as well as the matrix metalloproteinase-2, -9, -13, and -14 are expressed by osteoclasts and/or other bone cells and have been implicated in the turnover of bone and cartilage. To identify proteases that could act as activators of TRAP in bone, we report here that cathepsins K and L, in contrast to the matrix metalloproteinases, efficiently cleaved and activated recombinant TRAP in vitro. Activation of TRAP by cathepsin K/L was because of increases in catalytic activity, substrate affinity, and sensitivity to reductants. Processing by cathepsin K occurred sequentially by an initial excision of the loop peptide Gly143–Gly160 followed by the removal of a Val161–Ala162 dipeptide at the N terminus of the C-terminal 16-kDa TRAP subunit. Cathepsin L initially released a shorter Gln151–Gly160 peptide and completed processing at Ser145 or Gly146 at the C terminus of the N-terminal 23-kDa TRAP subunit and at Arg146 at the N terminus of the C-terminal 16-kDa TRAP subunit. Mutation of Ser145 to Ala partly mimicked the effect of proteolysis on catalytic activity, identifying Ser145 as well as Asp146 (Funhoff, E. G., Ljusberg, J., Wang, Y., Andersson, G., and Averill, B. A. (2001) Biochemistry 40, 11614–11622) as repressive amino acids of the loop region to maintain the TRAP enzyme in a catalytically latent state. The C-terminal sequence of TRAP isolated from rat bone was consistent with cathepsin K-mediated processing in vitro. Moreover, cathepsin K, but not cathepsin L, co-localized with TRAP in osteoclast-resorptive compartments, supporting a role for cathepsin K in the extracellular processing of monomeric TRAP in the resorption lacuna.

TRAP-resistant acid phosphatase (TRAP),1 also known as type 5 acid phosphatase (EC 3.1.3.2) or uteroferrin, belongs to the purple acid phosphatase (PAP) subfamily of the non-heme dinuclear metallophosphatas (1–3). The metals of the catalytic center of all PAPs consist of a common ferric ion and a divalent metal cation in an active enzyme, where mammalian PAPs characteristically contain a redox-active iron in the M(II) site (4–6).

The TRAP enzyme is abundantly expressed by bone-resorbing cells, osteoclasts, and certain subpopulations of monocytes/macrophages and dendritic cells (7–10). The precise role of osteoclastic TRAP is not fully understood, but studies on TRAP knock-out mice showed disturbed endochondral ossification with decreased resorptive activity of osteoclasts (11, 12), whereas overexpression of TRAP was associated with increased bone turnover (10). Different functions have been suggested for TRAP, e.g. as an osteopontin phosphatase (13–15), generation of reactive oxygen species (16–19), iron transport (20–24), and as a growth/differentiation factor for hematopoietic (25) and osteoblastic (26) cells.

Mammalian PAPs are synthesized as 35–37-kDa monomers but are commonly isolated from tissues as proteolytically cleaved two-subunit forms consisting of a 23-kDa N-terminal domain disulfide-linked to a 16-kDa C-terminal domain. The monomeric form exhibits properties of a proenzyme with low phosphatase activity that is converted to a high activity, two-subunit form upon proteolytic cleavage in the intervening loop domain with either serine proteases, e.g. trypsin or chymotrypsin (27), or members of the cathepsin family (28, 29). Mutagenesis studies suggested that proteolysis removes or alters repressive interactions between loop amino acids and active site residues because replacement of Asp146 of the exposed loop domain with Ala resulted in activation of unproteolyzed TRAP (30).

Several lines of evidence indicate a role for cathepsin K in bone resorption. Cathepsin K is highly expressed in osteoclasts near the ruffled border membrane and has been shown to participate in osteoclast-mediated degradation of the sub-osteoclastic collagenous bone matrix (31–36). Cathepsin K-overexpressing mice showed an increased turnover of metaphyseal trabecular bone (37), whereas cathepsin K knock-out mice displayed an osteopetrotic phenotype because of impaired matrix degradation (38–40). Besides cathepsin K, also cathepsins B, H, L, and S are expressed by osteoclasts and could participate in bone resorption (35, 41–43). Deletion of the cathepsin L gene was associated with reduction of trabecular bone and a diminished resorptive response following ovariec- tomy (44). In addi-

FITC, fluorescein isothiocyanate; FPLC, fast protein liquid chromatography; rec, recombinant; DTT, diithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcriptase; qPCR, quantitative PCR.
tion to cathepsins, different matrix metalloproteinases (MMPs), e.g. MMP-2, -9, -13, and -14, have been implicated in various aspects of skeletal development and turnover (45), including processes such as osteoclast recruitment and migration (46–49), post-osteoelastic cleaning of the resorption lacuna (50), and osteoblast survival (51).

In this study, several members of the cathepsin and MMP families with known association with the functional activities of osteoclasts were screened for cleavage and activation of rat recombinant TRAP, identifying cathepsin K and L as efficient activators of TRAP. In order to better understand the structural basis for proteolytic activation of mammalian PAPs, we also aimed to define the proteolytic cleavage sites for cathepsin L and K in the repressive loop domain. Finally, the in situ distribution of the cathepsins K and L was compared with that of TRAP in osteoclasts and resorptive subapartments to address the potential physiological relevance of the in vitro observations.

EXPERIMENTAL PROCEDURES

Materials

Proteases were supplied from the following sources: cathepsin L (human liver, 1000 U/mg), cathepsin K (human ileum, 1000 U/mg), MMP-2 from AnaWo Trading SA, (Wangen/Zurich, Switzerland); MMP-9, Roche Applied Science; MMP-13 and MMP-14, Invitrek (Berlin, Germany). Human mature cathepsin K was generated as described previously (31). Antibodies came from the following sources: alkaline phosphatase-conjugated goat anti-rabbit IgG from Sigma; horseradish peroxidase-conjugated goat anti-rabbit IgG from Bio-Rad (Gostrup, Denmark); FITC-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-rabbit IgG, and Cy3-conjugated donkey anti-goat IgG from Jackson ImmunoResearch (West Grove, PA). Goat anti-mouse cathepsin L was purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Rabbit anti-rat cathepsin K (52) was kindly provided by Dr. Hideaki Sakai, Nagasaki School of Dentistry, Sakamoto, Nagasaki, Japan. Rabbit anti-recombinant rat TRAP antibody (anti-recTRAP) was generated as described previously (53). Antiserum toward the exposed loop domain in TRAP was produced in rabbits using a synthetic peptide derived from the mouse TRAP sequence (1<sup>46</sup>PDFASQPQKMPRDLVGA(C)<sup>165</sup> as the immunogen (54). Restriction enzymes, DNA purification system, mutation-specific primers, and baculovirus expression system were purchased from Invitrogen.

Methods

Mutagenesis, Expression, and Purification of Recombinant TRAP

Site-directed Mutagenesis—The full-length 1.4-kb rat tartrate-resistant acid phosphatase cDNA (16) was cloned into pcMV5. Site-directed mutagenesis was performed using 5′–3′ MORPH site-specific plasmid DNA mutagenesis kit (5 Prime → 3 Prime, Inc., Boulder, CO). Primer used for specific mutagenesis of S145A was 5′-GCTGTTGTTGCAATGCA-GACGACTTGTGTC-3′; for Q152A was 5′-GACGACTTTGTCAGC-GCACTGGAAATTGC-3′. The underlined bases indicate changes compared with the wild-type sequence. The primer employed for phosphorylation was modified by T4 kinase according to the manufacturer's instruction. The recTRAP cDNA mutants were verified by DNA sequence analyses (Cybergene AB, Novum Research Park, Huddinge, Sweden).

Construction of Baculovirus Expression Vectors and Recombinant Baculovirus—Wild-type or mutant rat TRAP cDNAs were cloned into baculovirus expression vector pFastBac1 using the EcoRI site in the donor plasmid. The correct orientation was determined by PstI cleavage and further confirmed by DNA sequencing. The pFastBac1 donor vectors containing wild-type or mutant TRAPs were transformed into DH10Bac cells for homologous recombination with bacmid. Recombinants were selected on Luria agar plates containing antibiotics (50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline), 100 μg/ml Blue-gel (halogenated indolyl-β-galactoside), and 40 μg/ml isopropyl 1-thio-β-D-galactopyranoside and confirmed by PCR.

Virus Amplification—Spodoptera frugiperda (Sf9) cells were cultured in SF900 II SFM medium and transfected with purified bacmids using the transfection reagent Cellfectin (Invitrogen). After transfection, the cells were kept and incubated at 27 °C for 3 days. The medium, which constitutes the scale up-1 virus stock, was harvested and stored at 4 °C. This scale up-1 virus stock was used to infect a 25-cm<sup>2</sup> flask seeded with 1 × 10<sup>6</sup> cells, and the culture was incubated at 27 °C for 5 days. The supernatant constitutes the scale up-2. The recombinant virus stock scale up-2 was amplified once more by infection of 100 ml of Sf9 cells seeded at a density of 1 × 10<sup>6</sup> cells/ml with 3 ml of scale up-2, and after 5 days, high titer virus stock (10<sup>6</sup> pfu/ml) was harvested. The high titer stock virus was used in protein production.

Protein Expression and Purification—Purification of rat bone TRAP and recTRAP expression in Sf9 cells, purification, and protein determination were carried out as described previously (55).

Proteolytic Cleavage of TRAP

Small Scale Proteolytic Cleavage of TRAP—Proteolytic activation of TRAP was performed with the following proteases and incubation conditions: cathepsin K, L, and B and recTRAP, 7 ng/μl, 4 mol of protease/mol of TRAP, 50 mM NaAc, 5 mM EDTA, 0.1% Triton X-100, pH 6.5, 2 mM DTT. All proteases were prerurred in 2 mM DT for 7 min before the addition to the incubation solution, and the protease digestions were performed at 37 °C for 2 h. For MMP-2 and MMP-9, recTRAP, 10 ng/μl, or collagen type IV, 100 ng/μl, was used as control was incubated with MMP-2, 10 ng/μl (0.6 mol of MMP-2/mol of TRAP), or MMP-9, 20 ng/μl, 4 microunits/μl (0.9 mol of MMP-9/mol of TRAP), at 37 °C for 24 h in 50 mM Tris-HCl, 0.2 mM NaCl, 10 mM CaCl<sub>2</sub>, and 2 mM HgCl<sub>2</sub>, pH 7.2. For MMP-13 and MMP-14, recTRAP, 10 ng/μl, were incubated with MMP-13, 11.3 ng/μl, 5 microunits/μl (2 mol of MMP-13/mol of TRAP), or MMP-14, 11.8 ng/μl, 1.7 microunits/μl (2 mol of MMP-14/mol of TRAP) at 37 °C for 24 h in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, and 0.1% Triton X-100.

Large Scale Cathepsin K, Cathepsin L, and Trypsin Cleavage of TRAP Prior to FPLC Separation—recTRAP was incubated at 37 °C for 45 min with cathepsin L or cathepsin K with the final concentrations: recTRAP 50 ng/μl; 0.04, 0.2, 0.28, and 1.0 mol of protease/mol of TRAP, 50 mM NaAc, 1 mM EDTA, pH 5.5, 2 mM DTT. recTRAP was digested with trypsin at the final concentrations: recTRAP 35 ng/μl; 0.04, 0.2, 0.28, and 1.0 mol of trypsin/mol of TRAP 50 ng/μl, 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.1% Triton X-100, at room temperature for 45 min. All proteolytic digestions were stopped with the protease inhibitor mixture Complete (Roche Applied Science).

TRAP Activity Assay

Phosphatase activity was assayed in 96-well plates (Greiner Labortechnik, Frickhausen, Germany) with pNPP as substrate in 150 μl of incubation medium, pH 5.8, with final concentrations as follows: 10 mM pNPP, 1 mM acetic acid, 0.1 mM Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>, 0.1 mM NaAc, 0.15 mM KCl, 10 mM disodium tartrate, 0.1% (w/v) Triton X-100. The p-nitrophenol liberated after 1 h of incubation at 37 °C was converted to p-nitrophenol by addition of 100 μl of 0.3 mM NaOH. The absorbance was measured at 405 nm (ε = 18.9 nmol<sup>-1</sup> cm<sup>-1</sup>) with a Spectrawave 250 spectrophotometer (Molecular Devices, Sunnyvale, CA). 1 unit of TRAP activity corresponds to 1 μmol of p-nitrophenol formed per min. Kinetic experiments were performed with p-NPP concentrations varying from 0.2 to 50 mM K<sub>m</sub>, and V<sub>max</sub> values were calculated by regression analysis of Lineweaver-Burk plots using the Origin software (OriginLab Corp., Northampton, MA). pH optimum was determined in 0.1 mM NaAc with pH values ranging from pH 4.0 to 6.7. Sensitivity to reduction was performed with concentrations of sodium ascorbate ranging from 0.5 to 100 mM.

Western Blotting

SDS-PAGE was run under reducing conditions essentially as described by Laemmli, and proteins were electroblotted to Immuno-PVDF membranes (Bio-Rad). General procedure for all membranes was as follows. To minimize nonspecific binding, immunoblots were preincubated for 2 h at room temperature or overnight at 4 °C in 1% Tween 20 in TBS (20 mM Tris, pH 7.5, 500 mM NaCl). The membranes were then incubated with primary antibody for 1 h at room temperature, washed three times for 10 min each in TBST (TBS + 0.05% Tween 20) before incubation with secondary antibody, washed, and developed. Probing and development was as follows. Immunostaining of TRAP was performed with rabbit anti-recTRAP antisemur (diluted ×100) and alkaline phosphatase-conjugated goat anti-rabbit IgG as the secondary antibody (diluted ×500). Development was performed with nitro blue tetrazolium chloride/5-bromo-4-chloroindol-3-yl phosphate p-toluidine salt (Bio-Rad).
Proteolytic Processing of TRAP by Cathepsin K

FPLC Separation of TRAP Proteolytic Fragments

Separation of proteolytic TRAP fragments was performed according to the methods described previously(56) using a ΔKTAPurifier™ 10 FPLC system at 4 °C with a heparin column (flow rate 2 ml/min) equilibrated with 20 mM Tris-HCl, pH 7.2, 0.1 M NaCl, 0.005% Triton X-100 (w/v). TRAP was eluted by a linear gradient of NaCl from 0.1 to 1 M NaCl (200 mM Tris-HCl, pH 7.2, 0.005% Triton X-100 (w/v) (12.5 column volumes) and collected in 0.75 ml fractions.

Protein Sequencing

N-terminal Sequence Analysis—Samples in solution were applied to a Procise HT sequencer (Applied Biosystems) for Edman degradation. C-terminal Sequence Analysis—The sample was treated with phenylisocyanate to block the N terminus and the e-amino group of lysines. The sample was thereafter mounted in the reaction cartridge of a Procise C sequencer instrument (Applied Biosystems). The sequencer was operated essentially according to the manufacturer’s recommendations. Briefly, after initial activation of the C terminus with acetic anhydride, the sample was treated with tetrabutylammonium thiocyanate to form the thiodydroxyn inner phosphate. Subsequently, this derivative was 8-alkylated by bromomethyl-naphthalene to yield an alkyl amidophenyl ribonucleotide derivative that is cleaved by trifluoroacetic acid and analyzed by reverse phase (C18) chromatography (detection at 257 nm). This was repeated with exclusion of the acetic anhydride activation step used in the initial cycle only. For testing, myoglobin was used to check the instrument performance, and alkylated thiodyhydroxyn amino acid standards were always run before analysis of sample cycles. At least three cycles were performed for N- and C-terminal sequencing.

Relative Quantification of Cathepsin K and L mRNA

Reverse Transcripase-PCR—For RT-PCR, the long bones from 3-week-old Sprague-Dawley rats were immersed in liquid nitrogen immediately after dissection and stored at −70 °C. Total RNA was extracted using the TotalTALLY RNA™, total RNA isolation kit according to the protocol of the manufacturer (Ambion, Austin, TX). After extraction, the RNA was treated with 5 units of DNase I (Invitrogen) for 30 min at room temperature. The reverse transcription reaction was performed with 5 μg of total RNA and 200 units of Superscript™ II reverse transcriptase (Invitrogen) at 42 °C, using dT20 as primer (Invitrogen), according to the protocol of the manufacturer. In negative controls, reverse transcription was excluded from the reaction mixture. PCR amplifications of cathepsin L and cathepsin K were performed on aliquots of cDNA corresponding to 160 ng of total RNA using 2.5 units of TaqDNA polymerase in the presence of 1 M NaCl in 20 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% Triton X-100 (w/v) (12.5 column volumes) and collected in 0.75-m1 aliquots.

FPLC Separation of TRAP Proteolytic Fragments

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Immunofluorescence Labeling

Long bones from 3-week-old Sprague-Dawley rats were immersed in 4% paraformaldehyde in phosphate-buffered saline immediately after dissection. The bones were decalcified with 20% formic acid for 2 days prior to embedding in paraffin. Sections (4 μm) were adhered to slides (Menzel-Glaser; Brandenburg, Germany) and dried at 60 °C. After deparaffinization and rehydration, all slides were microwaved-treated by boiling in 1× EDTA for 5 min and then blocked in 0.1% BSA-c (Auron, Seligenstadt, Germany) for 20 min in a humidified chamber at room temperature. For immunostaining of TRAP and cathepsin L, sections were incubated with rabbit anti-recTRAP (diluted ×500) (53) for 60 min at room temperature and washed with TBS (50 mM NaCl, pH 7.6, 150 mM NaCl) three times. The sections were then incubated with swine anti-rabbit IgG (diluted ×100) for 30 min to prevent nonspecific binding of cathepsin K secondary antibody. incubation with rabbit anti-cathepsin K antibody (diluted ×200) for 60 min was followed by washing as above and incubation with Cy3-conjugated donkey anti-rabbit-IgG (diluted ×50). For immunostaining of TRAP and cathepsin L, incubation with goat anti-mouse cathepsin L (diluted ×100) and rabbit anti-recTRAP antibody (diluted ×500) was performed for 60 min followed by washing in TBS. Sections were then incubated with FITC-conjugated donkey anti-rabbit-IgG (diluted ×100) and Cy3-conjugated donkey anti-goat-IgG (diluted ×250) and then washed. Slides were mounted with VECTASHIELD Mounting Media (DAKO, Glostrup, Denmark), and examination of immunostainings was done by using a Leica DM IRB microscope with a Leica TCS NT ArKr laser.

Immunohistochemistry

Immunostaining of TRAP was performed as described previously (54). In brief, sections were stained using the automated staining system Horizon (Dako, Glostrup, Denmark) using rabbit anti-rat TRAP antibody, recognizing both monomeric and two-subunit TRAP (53), and diluted 1:400. As secondary antibodies and developing solutions, ChemMate detection kit peroxidase/5,3′-diaminobenzidine rabbit/mouse (Dako, Glostrup, Denmark) was used.

Ultrastructural Immunohistochemistry

The tissue used for immunoelectron microscopy was fixed by immersion in a 0.1 m phosphate-buffered (PB) mixture of 2% paraformaldehyde with 0.5% glutaraldehyde and decalcified in EDTA (triplax II) as described previously (12). Tibia was cut in smaller pieces and dehydrated in methanol at low temperature in a Leica AFS (Leica, Heerbrugg, Switzerland) and embedded in lowcryl K11M (Chemische Werke Loewi GmbH, Waldkirch, Germany) at low temperature as described previously (58). Ultrathin sections were cut and placed on carbon/Formvar-coated nickel grids. To block nonspecific binding, the grids were incubated with 2% bovine serum albumin (Sigma, fraction V) (0.1 m PB buffer and subsequently with the primary antibodies against recombinant TRAP (diluted 1:500), the TRAP loop peptide (diluted 1:500), or cathepsin K (diluted 1:1000), respectively. The bound antibodies were detected with protein A-coated 10 nm gold particles (Amersham Biosciences). The sections were subsequently contrasted and examined in a Tecnai 10 (Fei Co., The Netherlands) transmission electron microscope. Osteoclasts were identified by obvious clear zone and ruffled border and a considerable number of mitochondrial profiles.
Particles in the bone matrix (Table II) at the ruffled border. The background (Table II) was used for quantitative analysis. The contact area between ruffled border and bone matrix was divided into two compartments parallel to the bone-ruffled border, each 2 μm wide. The first compartment is referred to as ruffled border (Table II), i.e. the intra-cellular area of the ruffled border. The other area represented the bone compartment is referred to as ruffled border (Table II), i.e. the intra-cellular area of the ruffled border. The background (Table II) was calculated from images taken in the central parts of the bone. Gold particles were counted, and the corresponding areas were measured by a semiautomatic interactive image analyzer (Videoplan, Zeiss, Oberkochen, Germany) giving the concentration as number of gold particles/μm². The data (Table II) represent analysis from 24 different osteoclasts from three animals.

RESULTS

Cathepsin Specificity for Proteolytic Processing and Activation of TRAP—We have shown previously that the cysteine proteases papain, cathepsin B, and cathepsin L can cleave and activate the latent monomeric 37-kDa form of rat recombinant TRAP to generate a more active two-subunit variant resembling the most abundant TRAP form detected in various tissues, including bone (28, 29, 53). Because such limited proteolytic cleavage could represent an important and physiologically relevant mechanism for determining when and where the enzyme should be active during osteoclastogenesis and the resorptive event, it was of importance to compare the major cathepsins known to be expressed in osteoclasts, e.g. cathepsins B, L, and K, for their preference for cleavage and activation of TRAP. Monomeric recTRAP was subjected to proteolytic digestion by the cysteine proteases cathepsin K, L, S, or B at 4 mol of protease/mmol of recTRAP for 2 h at 37 °C (Fig. 1). Cleavage was assessed by disappearance of the 37-kDa monomeric TRAP and appearance of 20–23- and 16-kDa fragments representing the N- and C-terminal domains, respectively. TRAP enzymatic activity was determined using pNPP as substrate and assessed whether these matrix metalloproteinases were capable of cleaving and activating TRAP. Western blot analysis showed that the 37-kDa recTRAP band was degraded to some extent following incubation with the different MMPs (Fig. 2). However, the sizes of cleavage products was different from those generated by the cathepsins, and most importantly, no enzymatic activation was observed. As controls for proteolytic activity of the MMPs, collagen IV was digested with MMP-2 and MMP-9 and gelatin with MMP-13 and MMP-14, and the expected cleavage products were visualized by protein staining following SDS-PAGE (data not shown). These results indicate that the matrix metalloproteinases-2, -9, -13, and -14 are unable to activate recTRAP.

Matrix Metalloproteinases-2, -9, -13, and -14 Are Unable to Activate recTRAP—Besides cathepsins, members of the MMP family have been indicated in bone resorption and turnover, e.g. MMP-2, -9, -13, and -14 (45). Therefore, it was important to assess whether these matrix metalloproteinases were capable of cleaving and activating TRAP. Western blot analysis showed that the 37-kDa recTRAP band was degraded to some extent following incubation with the different MMPs (Fig. 2). However, the sizes of cleavage products was different from those generated by the cathepsins, and most importantly, no enzymatic activation was observed. As controls for proteolytic activity of the MMPs, collagen IV was digested with MMP-2 and MMP-9 and gelatin with MMP-13 and MMP-14, and the expected cleavage products were visualized by protein staining following SDS-PAGE (data not shown). These results indicate that the matrix metalloproteinases-2, -9, -13, and -14 are not readily cleaving and activating the TRAP protein in vitro.

pH Optimum and Reducing Properties after Cathepsin K Cleavage—pH optima for PAPs have been reported earlier in the range from pH 4.5 to 6.5. Monomeric recombinant rat
TRAP exhibited a broad pH optimum of around 4.5–5.5 (Fig. 3A), which increased by 1 pH unit after cleavage of the enzyme with papain (28). After cathepsin K cleavage, the pH optimum of monomeric recTRAP shifted to 5.5–6.0, concomitant with significantly increased enzymatic activity (Fig. 3A). The two-subunit TRAP from rat bone exhibited a similar pH profile and activity level as cathepsin K-digested recTRAP and was not altered by incubation with cathepsin K.

Mammalian PAPs are redox-sensitive di-iron enzymes, where the inactive di-iron enzyme can be converted to the active mixed valent ferri/ferrous state by a one-electron transfer provided by a reducing agents such as ascorbate (4). We analyzed the response of monomeric recombinant and bone-derived rat TRAP to various ascorbate concentrations in the range from 0.5 to 100 mM before and after cathepsin K digestion (Fig. 3B). For untreated recTRAP, even ascorbate concentrations as high as 100 mM minimally activated the enzyme, whereas the activity of cathepsin K-cleaved recTRAP was significantly augmented with a maximum reached at 10 mM. Most interestingly, no difference in TRAP activity was noted between the uncleaved and cathepsin K-cleaved forms in the absence of reductants, suggesting that proteolysis does not activate the enzyme per se but potentiates the sensitivity of the redox-active iron to reductants. The two-subunit TRAP from rat bone showed a similar response to ascorbate as cathepsin K-digested recombinant TRAP.

**Proteolytic Processing of TRAP by Cathepsin K**

**Separation and Characterization of Two-subunit TRAP Variants**—Heparin ion-exchange FPLC has proven useful to separate a proteolytically cleaved two-subunit form of rat TRAP from the intact monomeric TRAP (55). In order to assess fragmentation patterns and to purify sufficient amounts of cleaved TRAP for protein sequence analysis, recTRAP was digested with trypsin, cathepsin L, or cathepsin K at different protease to TRAP ratios (0.04–1 mol/mol) and applied to the heparin column (Fig. 4). Samples were eluted by a linear sodium chloride gradient from 0.1 to 1.0 M (56). Trypsin-digested recTRAP eluted as a single peak at approximately 0.5 M NaCl just as the uncleaved recTRAP used as control (Fig. 4a), indicating that trypsin cleavage does not alter the surface charge properties of the TRAP protein. In contrast, native two-subunit TRAP purified from rat bone eluted in three separable peaks, with the two major peaks at 0.65 and 0.75 M NaCl, respectively (Fig. 4b). The elution profiles of cathepsin L (Fig. 4c) and cathepsin K (Fig. 4d)-digested samples were similar to that of bone TRAP with two peaks of TRAP activity (labeled L2, L3 for cathepsin L-, and K2, K3 for cathepsin K-cleaved recTRAP) at 0.65 and 0.75–0.8 M NaCl. With increasing protease to TRAP ratio, a transition from peak 2 to 3 occurred, suggesting that peak 2 represents an intermediate form.

Western blot analysis of the TRAP activity peaks eluted from the heparin column was performed in order to verify proteolytic cleavage and to determine the sizes of the N- and C-terminal fragments formed (Fig. 5). Blots were probed with a polyclonal antibody generated against the intact recombinant rat TRAP protein, recognizing both the monomeric and two-subunit forms of recombinant and bone TRAP, respectively (Fig. 5, a and b). In addition, a novel antibody raised to a peptide covering amino acids 146–162 in rat and mouse TRAP sequence (Fig. 5, c and d) was used in order to verify the presence or absence of the loop peptide sequence in the proteolyzed variants of recTRAP. The loop-specific antibody reacted to the monomeric TRAP (lane C in Fig. 5c) but recognized neither the N-terminal 23- nor the 16-kDa C-terminal fragments in rat bone TRAP (Fig. 5a). In trypsin-digested recTRAP (Fig. 5, b and c, lane T1), two proteolytic bands at 25 and 16 kDa was recognized by the total TRAP antibody (Fig. 5b, lane T1) but only the larger fragment was detected by the loop antibody (Fig. 5c, lane T1), suggesting the epitope was still present on the N-terminal fragment. Peaks L2 and L3 from cathepsin L-cleaved recTRAP contained two proteolytic fragments of 23 and 16 kDa (Fig. 5, b and c), but just as with rat bone TRAP the loop antibody failed to recognize any of these fragments (cf. Fig. 5, b and c). A similar pattern, but with a slightly lower size of the 23-kDa bands, was noted for the peaks K2 and K3 from cathepsin K-cleaved recTRAP. The lack of recognition of the N-terminal fragments by the loop antibody along with a smaller size of N-terminal fragments following digestion with cathepsins L and K as compared with trypsin is consistent with excision of the antibody-binding loop peptide fragment.

**Protein Sequencing and Enzyme Kinetics of Proteolytic TRAP Variants**—The possibility that inactivation of the loop sequence epitope by cathepsin K/L digestion was due to excision of a part of the loop domain prompted verification by N- and C-terminal protein sequence analysis of the corresponding fragments. Sequence analysis was performed on the purified cleaved variants after incubation with a molar ratio of proteases to TRAP of 0.28.

The undigested recTRAP (peak C) did not result in any N-terminal sequence starting in the TRAP loop region as expected for the unproteolized recTRAP (Fig. 6). N-terminal sequencing of the trypsin-digested T1 peak (data not shown) indicated a single tryp tic cleavage site between Arg157 and Asp158, confirming previously published data (28). N-terminal sequencing of peak L2 yielded a sequence starting at Val161, whereas C-terminal sequencing provided a sequence starting with Ser150. This initial cleavage thus excises a peptide from
Gln151 to Gly160. Both the fragments were further digested, as indicated by the identification of Arg163 as the N-terminal amino acid, with Ser145 as the major (65%) and Gly143 as the minor (35%) C-terminal loop amino acid in peak L3. N-terminal sequences beginning at Val161 could be assigned for peak K2 and at Arg163 for peak K3. Cys142 was the single C-terminal amino acid identified in peaks K2 and K3. Thus, removal of an extended region of the loop domain by cathepsins K and L occurs sequentially. The N-terminal amino acids Val161 and Ala162 of the smaller 16-kDa fragment appears responsible for the different elution properties of the major cleaved forms from the heparin column. The N- and C-terminal sequences of peaks B2 and B3 from TRAP isolated from rat bone were identical to the sequences in K2 and K3, consistent with processing by cathepsin K in vivo. More importantly, cathepsins L and K differed in the processing at the C-terminal end of the larger 23-kDa fragment. Because this region contains putative regulatory amino acids, e.g. Asp146, suggested by site-directed mutagenesis to exert a strong repressive influence on the catalytic activity of the intact, uncleaved enzyme (30), the cata-

**FIG. 4.** Heparin FPLC elution profiles of TRAP after proteolytic digestion with trypsin, cathepsin L, or cathepsin K. recTRAP was digested with trypsin, cathepsin L, or cathepsin K with various protease to TRAP ratios (0.04, 0.2, 0.28, and 1 mol of protease/mol of TRAP). The proteolytic digests were applied to a heparin FPLC column and eluted with a 0.1–1 M sodium chloride gradient in 20 mTris-HCl, pH 7.2, 0.005% Triton X-100. a, elution profile of undigested recTRAP control (○) with a main peak C; trypsin-digested recTRAP (0.04 (●), 0.2 (▲), and 1 (□) mol of protease/mol of TRAP) with a main peak T1. b, native rat bone TRAP with peaks labeled B1, B2, and B3. c, cathepsin L-digested recTRAP (0.04 (●), 0.2 (▲), 0.28 (▲), and 1 (□) mol of protease/mol of TRAP) with peaks labeled L1, L2 and L3. d, cathepsin K-digested recTRAP (0.04 (●), 0.2 (▲), 0.28 (▲), and 1 (□) mol of protease/mol of TRAP) with peaks labeled K1, K2, and K3. Proteolysis and FPLC conditions were as described under “Materials and Methods.”

**FIG. 5.** Western blot analysis of heparin FPLC elution peaks of trypsin-, cathepsin L-, and cathepsin K-digested TRAP. 125 ng of recTRAP or bone TRAP was separated by a 12% SDS-PAGE under reducing conditions and blotted onto a PVDF membrane. a, bone TRAP was probed with anti-recTRAP antibody (1st lane) and anti-TRAP loop peptide antibody (2nd lane) as the primary antibody (Ab). b, anti-recTRAP antibody; c, anti-TRAP loop peptide antibody was used as primary antibody. b and c, lanes C, T1, L2, L3, K2, and K3 represent pools from the corresponding FPLC elution peaks. d, the sequences of the mouse and rat TRAP loop region (amino acid numbers (aa nr) 145–164). The mouse TRAP loop peptide sequence (amino acids 146–162) was used to generate antibody against the loop domain. (Rat TRAP numbering is according to Ref. 66.)
lytic activity and affinity constants of the different proteolytic cleaved forms were determined.

The $V_{\text{max}}$ and $K_m$ values were calculated by regression analysis of Lineweaver-Burk plots using pNPP concentrations from 0.2 to 50 mM at pH 5.8 in the presence of reductants. The unproteolyzed recTRAP control (C) had a specific activity of 198 units/mg and a $K_m$ of 4.6 mM (Fig. 6). The T1 peak after trypsin digestion exhibited similar $K_m$ (5.3 mM) as the unproteolated control, with 2.4-fold increased activity (data not shown).

The catalytic activity of the L2 and L3 peaks resulting from cathepsin L digestion was 5–6-fold increased compared with the control; however, the affinity constants differed considerably with $K_m$ values of 3.1 and 0.8 mM for the L2 and L3 peaks, respectively. The K2 and K3 peaks resulting from cathepsin K cleavage demonstrated a further activity increase with a 13–14-fold activation in K2 and K3. The apparent $K_m$ values for K2 (0.6 mM) and K3 (0.5 mM) were in the same range as for the L3. By correlating the kinetic analysis to the sequence data, increases in catalytic activity occur by sequential cathepsin L- and K-assisted C-terminal shortening of the loop sequence associated with the 23-kDa fragment.

**Mutagenesis of the Selected Loop Amino Acids**—As seen in Fig. 6, cleavage with cathepsin L increased TRAP activity 5–6-fold, whereas cathepsin K-cleaved TRAP exhibited a further 2.5-fold increase in specific activity compared with the control.

**Table I**

|   | $k_{\text{cat}}$ (s$^{-1}$) | $\text{- Fold activation}$ | $K_m$ (mM) | $k_{\text{cat}}/K_m$ × 10$^3$ | $\text{- Fold activation}$ |
|---|-----------------|-----------------|----------|------------------|-----------------|
| Wild-type TRAP | 279 (18) | ×1 | 2.0 (0.2) | 142 (12) | ×1 |
| Q151A | 235 (8) | ×0.8 | 3.3 (0.4) | 72 (6) | ×0.5 |
| Q152A | 424 (59) | ×1.5 | 3.1 (0.3) | 136 (22) | ×1.0 |
| S145A | 842 (79) | ×3.0 | 2.2 (0.1) | 378 (35) | ×2.7 |

Numbers in parentheses are S.D. values $n = 2$.

The possible involvement of Gln151, Gln152, and Ser145 in maintaining the monomeric TRAP in a catalytically repressed state was addressed by expressing alanine mutants, which were purified to apparent homogeneity and subjected to kinetic analysis (Table I). Unproteolyzed Q152A and S145A showed a 1.5- and 3-fold increase in catalytic activity, respectively, whereas the Q151A was not different from the wild-type enzyme. However, only the S145A showed an increased $k_{\text{cat}}/K_m$ value compared with wild-type recTRAP. Hence, Ser145 may be responsible for partly repressing the catalytic activity of the enzyme.

**Expression and Localization of TRAP and Cathepsin K and L in Rat Long Bone**—Because both cathepsin L and K efficiently converted latent monomeric TRAP into a highly active enzyme in vitro, we next addressed the important question of whether one or both of these enzymes could fulfill a role as an activator of TRAP in osteoclasts. Initially, we assessed the expression of these cathepsins in rat bone extracts at the mRNA and protein levels. By semi-quantitative RT-PCR (Fig. 7A, upper panel), a 246-bp cathepsin K fragment was detectable after 25 cycles and a 424-bp cathepsin L band after 30 cycles, indicating that cathepsin K is more highly expressed than cathepsin L at the mRNA level in rat long bones. Semi-quantitative real-time qPCR showed an approximately 40-fold higher relative mRNA expression level of cathepsin K compared with cathepsin L, normalized to GAPDH mRNA (Fig. 7A, lower panel). At the protein level, a major band corresponding to mature cathepsin K was detected (Fig. 7B), suggesting that cathepsin K predominantly exists as the active enzyme in bone. Three bands corresponding to (pre)procathepsin L, single chain cathepsin L and heavy chain cathepsin L were detected (Fig. 7B, lower panel), with (pre)pro-cathepsin L as the major form.

Next, immunofluorescence staining was performed on sections of rat long bone in order to examine the cellular distribution and co-localization of TRAP, cathepsin K and cathepsin L. TRAP (Fig. 8c) and cathepsin K (Fig. 8b) were expressed in a majority of osteoclasts on the trabecular bone surface. In double-stained sections (Fig. 8, c and d), it was apparent that TRAP and cathepsin K were co-localized intracellularly in osteoclasts. Double staining for TRAP (green) and cathepsin L (red) did not indicate co-localization in trabecular osteoclasts (Fig. 8e), but both proteins were present in a subset of mononuclear cells, possibly pre-osteoclasts not associated with bone, in the bone marrow cavity (Fig. 8f). These data are consistent with a role for cathepsin K in the proteolytic processing of TRAP in rat osteoclasts.

**Ultrastructural Distribution of TRAP and Cathepsin K in Osteoclasts**—In order to define more precisely the co-localization of TRAP and cathepsin K in different subcellular compartments in osteoclasts, ultrastructural immunocytchemistry.
was performed using the protein A-colloidal gold labeling method. Two different primary antibodies against TRAP, one selectively recognizing monomeric TRAP and the other antibody recognizing both monomeric and cleaved forms, i.e. total TRAP, were used, as well as an anti-peptide antibody to cathepsin K (52). The distribution of gold particles was assessed qualitatively (Fig. 9) in different cytoplasmic and extracellular osteoclast compartments and quantitatively (Table II) in different resorption areas of the osteoclast. The concentration of label for total, i.e. monomeric and cleaved, TRAP was slightly higher in the bone matrix compared with the ruffled border area (Fig. 9A). More importantly, the concentration of label for monomeric TRAP was close to background in the matrix and equal to total TRAP in the ruffled border area (Fig. 9B). Cathepsin K showed a similar distribution as monomeric TRAP in the resorption lacuna (Table I). Monomeric TRAP was also detected in Golgi cisternae and vesicles (Fig. 9C). Total TRAP (Fig. 9D), but not monomeric TRAP (Fig. 9E), was detected, often associated with amorphous luminal material, in large phagocytic vacuoles in the cytoplasm. Collectively, these data suggest that monomeric TRAP is transported in secretory compartments of osteoclasts and is secreted as the monomeric protein into the ruffled border, where cathepsin K could mediate processing to the cleaved form.

**DISCUSSION**

Mammalian purple acid phosphatases are synthesized as single N-glycosylated polypeptides with low enzyme activity (9, 59). Limited proteolytic cleavage in an exposed mid-region converts the protein to a two-subunit form, consisting of a 23-kDa N-terminal fragment disulfide-bonded to the 16-kDa C-terminal fragment, with enhanced catalytic activity (27–29). In this study, we have aimed to identify proteases that could be involved in processing and formation of the active TRAP enzyme in osteoclasts, one of the major TRAP-expressing cell types (9). Moreover, the structural basis for enzyme repression by the loop is largely unknown, although an interaction between the loop residue Asp156 and an unidentified active site residue was recently implicated by site-directed mutagenesis (30). Therefore, we were also interested in defining the critical residues of the repressive loop domain by correlating the sequence of different proteolytic fragments with their catalytic properties.

The exposed loop domain in the vicinity of the active site shown to repress catalytic activity extends from Gly143 of helix 7 to Gly156 at the beginning of helix 9 with γ-turns at Pro150 and Pro153 (numbering according to the rat TRAP sequence GenBankTM accession number M76110) (9, 60, 61). The serine protease trypsin cleaves in the C-terminal part of the loop to produce a nick between Arg155 and Pro156, which is conserved also in the mouse TRAP protein (62). Exclusively in the human enzyme, trypsin excises a tripeptide (158DVK160) between Arg157 and Leu161 (29). In addition, there are potential tryptic cleavage sites between Arg155 and Pro156 and between Lys154 and Met155 in the mouse (62) TRAP protein. In native two-subunit forms isolated from tissues, proteolytic cleavage results in a N-terminal Arg163 in the rat bone enzyme (28) or at Ala162 in the bovine spleen TRAP (64), arguing against the involvement of trypsin or trypsin-like proteases in the final proteolytic processing of TRAP in vivo.

We and others (27, 53) have noted that the reactivity of TRAP antibodies toward the native 23-kDa N-terminal fragment is relatively lower than to the monomeric precursor or to the C-terminal fragment. Hydropathy plots indicated a highly antigenic region at residues 155–157 (27), which corresponds to Met-Pro-Arg in the rat and mouse sequences and

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**Fig. 7. mRNA and protein expression of cathepsin K and L in rat long bone.** *A*, upper panel, semi-quantification of mRNA expression of cathepsin K and cathepsin L in rat long bone. PCR for cathepsin K and cathepsin L was run on reverse-transcribed mRNA from rat long bone, and aliquots were withdrawn at different cycles. *A*, lower panel, relative quantification of cathepsin K and L mRNA using real time qPCR. *** refer to statistical analysis using Student’s t test, n = 3, p ≤ 0.01 compared with cathepsin L. *B*, Western blot analysis of cathepsin K (upper panel) and L (lower panel) protein expression in rat long bone. 25 mg of bone homogenate was subjected to 12% SDS-PAGE. Blots were incubated with rabbit anti-rat cathepsin K or goat anti-mouse cathepsin L antibodies. The membranes were developed by nitro blue tetrazolium chloride/5-bromo-4-chloroindol-3-yl phosphate p-toluidine salt. One band was detected representing mature cathepsin K (28 kDa). Three bands were detected representing (pre)procathepsin L (35 kDa), single-chain cathepsin L (25 kDa), and heavy chain cathepsin L.

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**Proteolytic Processing of TRAP by Cathepsin K**

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**TABLE I.** Semi-quantification of mRNA expression of cathepsin K and L in rat long bone. PCR for cathepsin K and cathepsin L was run on reverse-transcribed mRNA from rat long bone, and aliquots were withdrawn at different cycles. *A*, upper panel, semi-quantification of mRNA expression of cathepsin K and cathepsin L in rat long bone. PCR for cathepsin K and cathepsin L was run on reverse-transcribed mRNA from rat long bone, and aliquots were withdrawn at different cycles. *A*, lower panel, relative quantification of cathepsin K and L mRNA using real time qPCR. *** refer to statistical analysis using Student’s t test, n = 3, p ≤ 0.01 compared with cathepsin L. *B*, Western blot analysis of cathepsin K (upper panel) and L (lower panel) protein expression in rat long bone. 25 mg of bone homogenate was subjected to 12% SDS-PAGE. Blots were incubated with rabbit anti-rat cathepsin K or goat anti-mouse cathepsin L antibodies. The membranes were developed by nitro blue tetrazolium chloride/5-bromo-4-chloroindol-3-yl phosphate p-toluidine salt. One band was detected representing mature cathepsin K (28 kDa). Three bands were detected representing (pre)procathepsin L (35 kDa), single-chain cathepsin L (25 kDa), and heavy chain cathepsin L.
Arg-Pro-Arg in the human and pig sequences, including or preceded by a potential tryptic cleavage site in the human, mouse, and pig TRAPs but not in the rat enzyme. Recently, a monoclonal antibody reacting to the monomeric TRAP 5a isoform, but not to the cleaved isoform 5b, was shown to bind a trypsin-sensitive epitope of the loop domain, corroborating the antigenic nature of this part of the protein (65). In order to facilitate detection of monomeric TRAP and cleavage fragments containing the loop, we generated a rabbit antibody toward the peptide sequence Ser145–Ala162 of the exposed loop (Fig. 5). Most interestingly, following trypsin digestion of the rat TRAP, strong reactivity was noted toward the resulting 23-kDa fragment as well as toward the monomeric form but not to the C-terminal 16-kDa fragment. Excision of the Gln151–Gly160 by cathepsin L was, however, associated with loss of loop antibody reactivity, consistent with localization of the epitope to this region. Because trypsin only cleaves between Arg157 and Asp158 in the rat TRAP (28) with no alteration of the epitope, whereas in human, mouse, and pig enzymes, an additional internal tryptic site is present between Arg155 and Pro156 or Lys154–Met155 with loss of antibody reactivity (65), our data suggest that the155(M/R)PR157 tripeptide constitutes the major antigenic epitope of the exposed loop domain. Moreover, because the size of the N-terminal 23-kDa fragment of rat recombinant TRAP following trypsin cleavage is larger by 1–2 kDa compared with the corresponding fragment from native two-subunit TRAP isolated from rat bone (28), additional trimming at the C terminus of this fragment is likely to occur in vivo.

In rat (28), bovine (27), pig (24, 27), and human (29) TRAP enzymes, trypsin digestion increases catalytic activity 2–4-fold with a minor influence on substrate affinity (29). The mechanism for stimulation of catalytic activity by trypsin is most likely indirect, because the distance between residues 155 and 157 and the iron center is >9Å and shows no hydrogen bonding interactions with active site residues or other loop residues (60, 61). Most interestingly, an extensive intrachain hydrogen bonding network is formed downstream of Arg157, suggesting that the enhancing effect of tryptic cleavage may be the result of increased flexibility of the truncated loop domain, thereby destabilizing critical interactions between loop residues and the active site.

We have shown previously that digestion of recombinant rat
TRAP with either cathepsin B (28) or cathepsin L (30) activated monomeric TRAP to levels comparable with that of native two-subunit TRAP isolated from rat bone. Because TRAP is abundantly expressed in osteoclasts, the main cell responsible for bone resorption, and cathepsin K is one of the major proteolytic enzymes synthesized by this cell, we were particularly interested in whether cathepsin K could process TRAP to a two-subunit form with similar structural and catalytic properties as the two-subunit TRAP isolated from rat bone (28). Our initial screening of several cathepsins with known expression in osteoclasts, i.e. cathepsin B, L, K, and S, indicated that both cathepsin L and K were more efficient than cathepsin B or S in cleaving and activating the monomeric TRAP. A dose-response experiment indicated that <0.1 mol of cathepsin K and around 0.3 mol of cathepsin L/mol of TRAP was sufficient to generate two-subunit TRAP with enhanced catalytic activity. For both enzymes, proteolytic processing proceeded through an intermediate fragment (L2, K2) that could be separated by using heparin ion exchange FPLC from the final L3/K3 cleavage products. Most interestingly, the L2/K2 and L3/K3 fragments differed in the N terminus of the 16-kDa fragment, suggesting that exposure of an N-terminal Arg increase the affinity of TRAP to heparin. Also the native-cleaved TRAP from rat bone (B2, B3) exhibited a very similar elution pattern as the cathepsin-digested monomeric TRAP, and moreover, a cathepsin K type of cleavage was apparent in two-subunit rat bone TRAP, consistent with processing by cathepsin K in TRAP expressed in rat bone.

Considerable differences in catalytic activity and substrate affinity was however associated with differential cleavage at the C-terminal end of the 23-kDa fragment. The L2 fragment lacking Gln151–Gly160 had 5.1-fold higher specific activity compared with the monomeric precursor, i.e. a relative 2-fold increase compared with trypsin cleavage (28). In the crystal structure of rat TRAP (Protein Data Bank code 1QHW) (60), the residues Gln151 and Gln152 are within hydrogen-bonding distance to the backbone carbonyls of Ser145 and Gln154, respectively (Fig. 10). Of these Gln residues, Gln151 may act as weak repressor through interaction with Ser145 supported by the tendency to slightly (1.5-fold) increased basal catalytic activity of the unproteolyzed Q151A mutant. The L3 fragment, lacking the Asp146–Ser156 sequence compared with the L2, exhibited a slight increase in catalytic activity but a significant increase in substrate affinity. This observation suggests that a critical residue controlling the repression of substrate affinity is present within this sequence. In a recent mutagenesis study (30), the importance of Asp146 in the control of both catalytic activity and substrate affinity was demonstrated. The carboxylate side chain of Asp146 is positioned sufficiently close for hydrogen bonding to the amido group of Asn91, which is a ligand to the Fe(II) ion (Fig. 10). Thus, by using the proteolytic approach of the present study, we suggest that the increase in substrate affinity with loss of the Asp146–Ser156 peptide is consistent with a repressive interaction of Asp146 on substrate affinity.

Another notable difference between the final cathepsin K and cathepsin L cleavage fragments, i.e. K3 versus L3, was observed in that a 2-fold higher catalytic activity was apparent in the former case. Comparison of the sequences indicated that either Ser145 or Asn144 was responsible for this difference. Consequently, we expressed a S145A mutant, which in its unproteolyzed form showed a 3-fold increase in catalytic activity. An interaction of the hydroxyl side chain of Ser145 with the Asp146–Ser150 sequence compared with the L2, exhibited a slight increase in catalytic activity but a significant increase in substrate affinity. This observation suggests that a critical residue controlling the repression of substrate affinity is present within this sequence. In a recent mutagenesis study (30), the importance of Asp146 in the control of both catalytic activity and substrate affinity was demonstrated. The carboxylate side chain of Asp146 is positioned sufficiently close for hydrogen bonding to the amido group of Asn91, which is a ligand to the Fe(II) ion (Fig. 10). Thus, by using the proteolytic approach of the present study, we suggest that the increase in substrate affinity with loss of the Asp146–Ser156 peptide is consistent with a repressive interaction of Asp146 on substrate affinity.

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and expressed in Sf9 insect cells as the recombinant TRAP of the present study (60). Most interestingly, in the structure of two-subunit recombinant TRAP (Protein Data Bank code 1QFC) (66), Ser145 is in a less favorable position for interaction with Asn91, consistent with a repressive role of this loop residue in the monomeric TRAP (60, 61).

Substrate specificity studies have shown that the primary specificity of papain-like cysteine proteinases is determined by S2-P2 interactions with a preference in cathepsin K for Pro or Ser with Gly or Ala in the P2 position, except with Ala in the P2 position. Cathepsin L cleaved at the N-terminal end of the loop, with a preferred Leu in the P2 position. Most interestingly, in N-telopeptide regions of α1(I) and α2(I) collagen chains, cathepsin K cleaves distal to a Gly or Ser residue with a hydrophobic Val or Leu in the P2 position (69), similar to what was observed for several of the cleavage sites for cathepsin K and L in the loop domain of TRAP.

Both cathepsin K and L are expressed in osteoclasts (71), although cathepsin K is more abundant than cathepsin L at the mRNA (72, 73) and activity (52) levels. Our RT-PCR and Western blot analysis on bone extracts and immunohistochemistry on bone tissue sections clearly indicated a higher expression of cathepsin K versus L in trabecular osteoclasts and, moreover, indicated that the majority of cathepsin K protein was processed to the active form and co-localized with TRAP in osteoclasts, whereas the majority of cathepsin L was present as the inactive precursor form and did not co-localize to a significant extent with TRAP in osteoclasts. Osteoclasts secrete TRAP (12, 74, 75) as well as cathepsin K (32, 76) into the resorption lacuna. In order to define the potential sites of cathepsin K-mediated processing of TRAP, we assessed the subcellular distribution of cathepsin K and the monomeric and proteolytically processed TRAP by ultrastructural immunocytochemistry.

For this purpose, we employed the loop antibody shown to recognize selectively the monomeric precursor of TRAP as well as the antibody generated toward the recombinant TRAP protein. Most interestingly, the distribution pattern with the loop antibody suggested that monomeric TRAP was transported in the secretory pathway and secreted in the ruffled border area of the osteoclast. Significant labeling for the total TRAP antibody in combination with low or absent labeling for the loop antibody is consistent with the presence of predominantly the processed, two-subunit TRAP in the bone matrix and in phagocytic vacuoles. In these vacuoles, TRAP labeling was more concentrated over what appeared as ingested matrix remnants, which indicate that a proportion of intracellular proteolytically processed TRAP derives from the bone matrix. Regarding cathepsin K, an enrichment of the enzyme was coincident with and at similar levels as the monomeric TRAP in the ruffled border area.

Recent studies demonstrated that cathepsin K is processed intracellularly in cultured human osteoclasts and is secreted as the active enzyme (36, 77). Thus, our data indicate that under normal bone turnover cathepsin K activates TRAP by excising the repressive loop subsequent to secretion of monomeric TRAP precursor into the acidic ruffled border area. The active TRAP enzyme then accumulates in the matrix and is partly internalized during matrix degradation.

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Proteolytic Excision of a Repressive Loop Domain in Tartrate-resistant Acid Phosphatase by Cathepsin K in Osteoclasts
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