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Protein-tyrosine phosphatase activity regulates osteoclast formation and function: Inhibition by alendronate

(bisphosphonates/osteoporosis/vanadate/bone resorption/cell fusion)

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ABSTRACT **Alendronate (ALN), an aminobisphosphonate used in the treatment of osteoporosis, is a potent inhibitor of bone resorption. Its molecular target is still unknown. This study examines the effects of ALN on the activity of osteoclast protein-tyrosine phosphatase (PTP; protein-tyrosine-phosphate phosphohydrolase, EC 3.1.3.48), called PTPε. Using osteoclast-like cells generated by coculturing mouse bone marrow cells with mouse calvaria osteoblasts, we found by molecular cloning and RNA blot hybridization that PTPε is highly expressed in osteoclastic cells. A purified fusion protein of PTPε expressed in bacteria was inhibited by ALN with an IC_{50} of 2 μM. Other PTP inhibitors—orthovanadate and phenylarsine oxide (PAO)—inhibited PTPε with IC_{50} values of 0.3 μM and 18 μM, respectively. ALN and another bisphosphonate, etidronate, also inhibited the activities of other bacterially expressed PTPs such as PTPσ and CD45 (also called leukocyte common antigen). The PTP inhibitors ALN, orthovanadate, and PAO suppressed *in vitro* formation of multinucleated osteoclasts from osteoclast precursors and *in vitro* bone resorption by isolated rat osteoclasts (pit formation) with estimated IC_{50} values of 10 μM, 3 μM, and 0.05 μM, respectively. These findings suggest that tyrosine phosphatase activity plays an important role in osteoclast formation and function and is a putative molecular target of bisphosphonate action.**

Osteoclasts, the multinucleated cells that resorb bone during bone remodeling and participate in Ca^{2+} homeostasis, are terminally differentiated cells derived from hemopoietic progenitors. The mature cells are formed by fusion of osteoclast precursors. Cells with osteoclastic features can be formed *in vitro* by coculturing bone marrow or spleen cells with osteoblasts or stromal cells in the presence of stimulators of bone resorption, such as interleukin 1, parathyroid hormone, or 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (1–3). During *in vitro* "osteoclastogenesis," osteoclast precursors differentiate into functional osteoclastic cells.

Protein-tyrosine phosphorylation plays an important role in the signal transduction pathways that control cellular growth, differentiation, and activity. Tyrosine phosphorylation levels are controlled by the dynamic equilibrium between the activity of protein-tyrosine kinases (PTK) and protein-tyrosine phosphatases (PTP) (4–11). The importance of PTK activity in osteoclast function was underscored by gene-deletion experiments in which disruption of the c-*src* protooncogene by homologous recombination induced osteopetrosis, a condition caused by reduced bone resorption due to impairment of osteoclast activity (12). It was subsequently shown that the expression of c-*src* was required for osteoclasts to be fully activated and to resorb bone (13). Furthermore, it was reported 12 years ago that the PTP inhibitor orthovanadate markedly inhibited parathyroid hormone-induced bone resorption in cultured tibiae (14).

Bisphosphonates (BP) exert their therapeutic effects by inhibition of osteoclast activity. They are used or being developed for the treatment of bone disorders that can benefit from inhibition of bone resorption, such as: hypercalcemia of malignancy, Paget disease, osteoporosis, and others. The BPs are organic pyrophosphate analogues in which two phosphates, are connected by a carbon (P—C—P) with various side chains (15). Alendronate (ALN, 4-amino-1-hydroxybutylidene-1,1-bisphosphonate) is a potent amino-BP used in for the treatment and prevention of osteoporosis (16–19). BPs, being organic pyrophosphate analogs, may influence biochemical pathways involved in phosphate metabolism, including protein phosphorylation and dephosphorylation.

We identified and cloned PTPε from mouse osteoclast-like cells and determined that ALN, orthovanadate, and phenylarsine oxide (PAO) inhibit the enzymatic activity of PTPε. We found that the PTP inhibitors suppress both in vitro osteoclast differentiation and bone resorption by freshly isolated osteoclasts, suggesting that PTP activity is essential for osteoclast function and could be a molecular target for BP action.

MATERIALS AND METHODS

Materials. Collagenase (no. 034-10533) was from Wako; dispase (no. 165859) was from Boehringer Mannheim; fetal bovine serum was from JRH Biosciences, Lenexa, KS; and other tissue culture reagents were from GIBCO/BRL. $1,25(OH)_2D_3$ was the gift of M. Uskokovich, Hoffmann–LaRoche. Tissue culture dishes were from NUNC, Inc., Naperville, IL. cDNA cloning was done by using the SuperScript Lambda system for cDNA synthesis and cloning, Life Technologies (Gaithersburg, MD). Sodium orthovanadate from Fisher Scientific was prepared according to the published protocol (21). BPs [ALN and etidronate (1-hydroxyethylidene-

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Abbreviations: ALN, alendronate; BP, bisphosphonate; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; FGF, fibroblast growth factor; FDP, fluorescein diphosphate; IGF, insulin-like growth factor; PAO, phenylarsine oxide; PTK, protein-tyrosine kinase; PTP, protein-tyrosine phosphatase; TRAP, tartrate-resistant acid phosphatase (uteroferrin); mPTPε and hPTPε, mouse and human PTPε; GST, glutathione *S*-transferase; FDP, fluorescein diphosphate.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession no. U40280).

1,1-bisphosphonate)] were synthesized at Merck or Gentili Laboratories.

Protein phosphatase type-1 (PP-1), its phosphopeptide substrate, Lys-Arg-Thr(*P*)-Ile-Arg-Arg, and the PTKs c-*src* and v-*abl* were from Upstate Biotechnology. PTK substrates and alkaline phosphatase (calf intestine) were from GIBCO/BRL. Pyrophosphatase (yeast) was from USB. Uteroferrin was a gift from S. U. Toverud. All other reagents were from Sigma.

Cells and Cultures. The mouse marrow coculture system was essentially as described (1). Osteoblasts that support bone marrow cell differentiation into osteoclasts were isolated from neonatal mouse calvaria (1). The osteoblasts were plated at 10,000/cm^2 in α-MEM containing 10% (vol/vol) fetal bovine serum and 10 nM 1,25$(OH)_2D_3$. BALB/c male mice (6 weeks old) were sacrificed under CO_2, and tibiae and femora were aseptically removed. The bone ends were cut off with scissors, and the marrow cavity was flushed with 1 ml of α-MEM by using a 27-gauge needle. The bone marrow cells were then filtered through 70-μm nylon mesh. Cells were centrifuged for 7 min at 300 $\times$ *g*, washed once with α-MEM, and finally resuspended; aliquots of 25,000/cm^2 were placed on the calvaria osteoblast cultures. Medium was replaced every two days. After 7-8 days, many multinucleated uteroferrin [tartrate-resistant acid phosphatase (TRAP)]-positive cells were present in these cultures.

For evaluation of cell fusion, cells are cultured in 24-well clusters. On day 7, cells are fixed with 10% formalin in phosphate-buffered saline for 10 min, permeabilized with 1:1 (vol/vol) acetone/ethanol for 1 min, and stained for TRAP for 10–20 min at room temperature. The TRAP staining solution contains 50 mM sodium acetate, 30 mM tartrate, 0.3 mg of fast red-violet LB per ml, and 0.1 mg of naphthol AS-MX (pH 5.0) per ml. The number of multinucleated TRAP$^+$ cells was counted microscopically in quadruplicate wells for each condition.

Human osteosarcoma and human giant cell tumor (osteoclastoma, GCT) and granulomatous tissues for RNA isolation were surgical specimens that were pathologically diagnosed and generously provided by T. A. Einhorn, M.D. (Mount Sinai Medical Center, New York).

Bone Resorption Assay. Six long bones were isolated from neonatal rats and were placed in 4.5 ml of 199 medium supplemented with 10% fetal bovine serum. The bones were dissected longitudinally, and cells were separated by scraping. The cell suspension was filtered through a nylon mesh. Circular slices of steer bone with a diameter of 4.4 mm and a thickness of 0.2 mm were sonicated, sterilized, and then hydrated in culture medium. The isolated cells (0.1 ml) were placed on the bone slices in 96-well tissue culture plates and incubated at 37°C under 5% CO_2/95% air with PTP or PTK inhibitors or vehicle for 24 hr. At the end of the experiment, the bone slices were sonicated, fixed with ethanol, and stained with methylene blue. The resorption pits were visualized by reflection light microscopy, and their number was determined (20).

Preparation of RNA and Hybridization. For RNA preparation, cells were cultured in 15-cm tissue culture dishes. To remove the osteoblastic cells, the cultures were washed twice with PBS and treated with collagenase/dispase (1 mg/ml each in PBS) at 37°C for 20 min. Cells were pipetted off the dish, and the plate was washed three times with PBS. In this process, most of the osteoblasts were removed from the plates, while the TRAP$^+$ multinucleated cells were left adhering to the tissue culture dishes (22). The RNA was then isolated by the guanidinium isothiocyanate method (23). Rat tibiae were dissected free of soft tissue, and the bone marrow was removed from the bones. RNA was isolated from these bones by the modified guanidinium hydrochloride method (24) and the guanidinium isothiocyanate method (23). Total RNA (20–30 μg) or poly(A)$^+$-selected RNA (2–5 μg) were electrophoresed on formaldehyde-containing agarose gels and transferred to nylon filters (Hybond N, Amersham, MA). A poly(A)$^+$ RNA blot of various human tissues was purchased from Clontech, CA. Poly(A)$^+$ RNA from cultured chicken osteoclasts was provided by Le Duong (Merck Research Laboratories). The hybridization solutions contained 50% formamide (Hybrisol I and II, Oncor, MD). After hybridization, the filters were washed in a solution of 2$\times$ SSC/0.1% SDS (1$\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) and finally in 0.2$\times$ SSC/0.1% SDS at 55°C and exposed to x-ray film (XAR-2, Kodak) with an intensifying screen at −70°C. The cDNA libraries of the *in vitro* generated osteoclasts were screened with hybridization solution containing 35% formamide (Hybrisol I and II) at 37°C. The final wash of the filters was with 1$\times$ SSC/0.1% SDS at 37°C.

Enzymatic Assays. The cDNA coding for the cytoplasmic region (amino acid residues 69–699) of PTPε was inserted in-frame into the bacterial expression vector pGEX-2TX (Pharmacia), thus forming a glutathione *S*-transferase (GST) PTPε fusion protein. The GST-PTPε fusion protein was isolated from the bacteria according to the Pharmacia protocol. No PTP activity was detected in the protein fraction purified from isopropyl β-D-thiogalactoside-induced bacterial cells transfected with vector DNA that contains the GST moiety without a PTPε insert or an insert coding for inactive PTPε protein. The enzymatic assays were performed in 96-well plates in 50 mM Mes (pH 5.65)/0.15 M NaCl/10% glycerol at ambient temperature. The cytoplasmic regions of PTPσ and CD45 were prepared and purified as GST fusion proteins as described (25, 26). Fluorescein diphosphate (FDP, Molecular Probes) was used as substrate (27). The dephosphorylation of FDP to fluorescein monophosphate produces an enhancement of fluorescence, which was monitored continuously by using a Millipore Cytoflour II plate reader, with an excitation wavelength of 485 nm (20-nm band width) and an emission wavelength of 530 nm (30-nm band width). The reaction rates were linear for at least 2 hr and were directly proportional to the amount of enzyme.

Inhibition of enzymes involved in phosphorylation were performed at the optimum pH and near K_m concentrations for the specific substrates. PTK and PP-1 assays were performed as described in the Upstate Biotechnology protocols. Pyrophosphatase activity was measured as free phosphate released after incubation for 10 min with 3 mM PP_i at 37°C in 100 mM Hepes/50 mM NaCl/1 mM EDTA, pH 7.1. Alkaline phosphatase activity was determined in a 7-min reaction at 37°C with 10 mM phosphotyrosine in 50 mM sodium acetate/100 mM Tris·HCl, pH 8.5. Uteroferrin activity was determined as described (28).

RESULTS

cDNA Cloning of Mouse PTPε (mPTPε) from Osteoclasts. To identify the PTPs expressed in osteoclasts, a cDNA library was prepared from mRNA isolated from a population of about 90% pure *in vitro* generated mouse osteoclasts. The phage λ cDNA library was screened under nonstringent hybridization conditions as described. As probe we used a cDNA fragment that codes for the cytoplasmic region of PTPα (29–32). Twenty positive clones were partially analyzed by restriction enzyme digestion and cDNA sequencing. In addition to cDNA clones for mouse PTPα, we found many clones that coded for a different PTP that was very similar to the human PTPε (hPTPε), that was cloned from a cDNA library of human placenta and was published while this work was in progress (32). We consider the PTP cloned from the osteoclasts as the mPTPε.

Sequence analysis revealed that mPTPε has 699 amino acid residues and has the structure of a transmembrane protein (Fig. 1). A putative signal peptide (underlined) is located next to the translation start site and a hydrophobic region between

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  1  MEPFCPLLLA SFSLSLARAG QGNDTTPTES NWTSTTAGPP DPGASQPLLT
        L     V G   P  TGS    E  A•D   E T  S             A

 51  WLLLPLLLLL FLLAAYFFRF RKQRKAVVSS NDKKMPNGIL EEQEQQRVML
           (LV)                    T S

101  LSRSPSGPKK FFPIPVEHLE EEIRVRSADD CKRFREEFNS LPSGHIQGTF
              Y               I        Q

151  ELANKEENRE KNRYPNILPN DHCRVILSQV DGIPCSDYIN ASYIDGYKEK
                              S      L

201  NKFIAAQGPK QETVNDFWRM VWEQRSATIV MLTNLKERKE EKCYQYWPDQ
                                 K                    H

251  GCWTYGNIRV CVEDCVVLVD YTIRKFCIHP QLPDSCKAPR LVSQLHFTSW
                                       Q      G

301  PDFGVPFTPI GMLKFLKKVK TLNPSHAGPI VVHCSAGVGR TGTFIVIDAM
                                 V                      S

351  MDMIHSEQKV DVFEFVSRIR NQRPQMVQTD VQYTFIYQAL LEYYLYGDTE
      A M A                          M

401  LDVSSLERHL QTLHSTATHF DKIGLEEEFR KLTNVRIMKE NMRTGNLPAN
            K     M G T          •

451  MKKARVIQII PYDFNRVILS MKRGQEFTDY INASFIDGYR QKDYFMATQG
                                    Y                    I

501  PLAHTVEDFW RMVWEWKSHT IVMLTEVQER EQDKCYQYWP TEGSVTHGDI
                  I                                       E

551  TIEIKSDTLS EAISVRDFLV TFKQPLARQE EQVRMVRQFH FHGWPEVGIP
       N          I        LN             V             I

601  AEGKGMIDLI AAVQKQQQQT GNHPITVHCS AGAGRTGTFI ALSNILERVK

651  AEGLLDVFQA VKSLRLQRPH MVQTLEQYEF CYKVVQDFID IFSDYANFK   699
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FIG. 1. Amino acid sequence of the mPTPε. The amino acid sequence of the mPTPε was deduced from the cDNA sequence. The residues of hPTPε that differ from mPTPε are indicated below the amino acid sequence of mPTPε. The black dots represent amino acid residues that are not present in hPTPε. The amino acid residues in parentheses represent additional residues found only in the hPTPε. The signal peptide (residues 1–13) and the transmembrane region (residues 48–68) are underlined by single and double lines, respectively. The two conserved PTP domains are boxed.

amino acid residues 47–70 is most likely a transmembrane domain (doubly underlined). Similar to hPTPε, mPTPε contains a cytoplasmic region with two tandem catalytic domains (boxed) and a relatively short extracellular domain of 45–47 amino acid residues. In the predicted structure of the mature protein, the extracellular region would comprise 25–27 amino acid residues with two potential N-glycosylation sites. As reported for hPTPε, a stop codon does not precede the first codon for methionine. Thus, although the 5′ untranslated sequences of hPTPε and mPTPε are different, the putative translation start site of mPTPε is identical to that of hPTPε. Overall, the mPTPε and the hPTPε share 93% of the amino acids, sequence differences being concentrated in the extracellular regions (Fig. 1). Compared to hPTPε, mPTPε has a few amino acid substitutions, a deletion of two amino acids in the transmembrane domain, and an additional amino acid residue in the short extracellular region.

Expression of PTPε in Osteoclasts. Hybridization of mPTPε cDNA to mRNA isolated from enriched populations of osteoclastic cells revealed high expression of mPTPε mRNA as a major transcript of about 5 kb and a minor transcript of about 2 kb (Fig. 2). These PTPε transcripts were also found in RNA isolated from a human giant cell tumor (osteoclastoma rich in multinucleated osteoclastic cells) and in a chicken osteoclast preparation (Fig. 2). PTPε expression was not detected in total RNA of osteosarcoma tumors that do not contain osteoclasts or in RNA isolated from granulomatous tissue that is rich in other cell types (not shown).

Expression of PTPε During *in Vitro* Osteoclast Differentiation. To study the relationship of PTPε to the osteoclast phenotype, we followed its expression during osteoclast differentiation. Total RNA was isolated from $1,25(OH)_2D_3$-treated cocultures of bone marrow and mouse calvaria osteoblastic cells at the time points shown in Fig. 3. PTPε mRNA was first detected on day 4 of coculture when $TRAP^+$ osteoclasts can first be seen (Fig. 3). The expression increased thereafter to reach maximal expression on days 6 and 7, when the number of osteoclastic cells peaked. No PTPε mRNA transcripts were detected when $1,25(OH)_2D_3$, which is essential for osteoclast differentiation, was omitted from the coculture or when osteoblasts were cultured alone, with or without $1,25(OH)_2D_3$. The PTPε mRNA was enriched several fold in RNA from an enriched preparation of osteoclasts. These observations indicate that the RNA for PTPε is found in

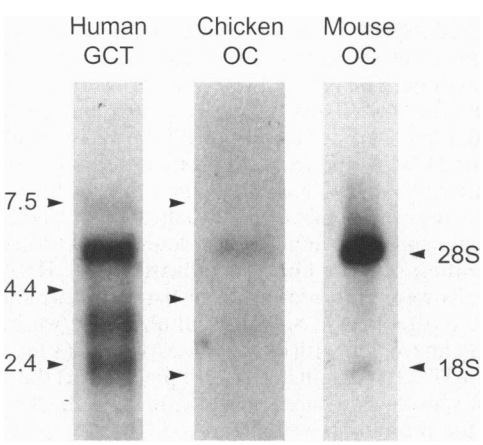

FIG. 2. Expression of PTPε RNA in osteoclasts. Total or poly(A)$^+$ RNA was isolated from giant cell tumor (GCT) tissue, cultured chicken osteoclasts (OC), and enriched populations of *in vitro*-generated mouse osteoclasts as described in text. GCT, 20 μg of total RNA from human giant cell tumor; chicken OC, 2 μg of poly(A)$^+$ RNA prepared from cultured osteoclasts isolated from chicken medullary bone; mouse OC, 2 μg of poly(A)$^+$ RNA prepared from *in vitro*-generated mouse osteoclasts purified from the coculture as described.

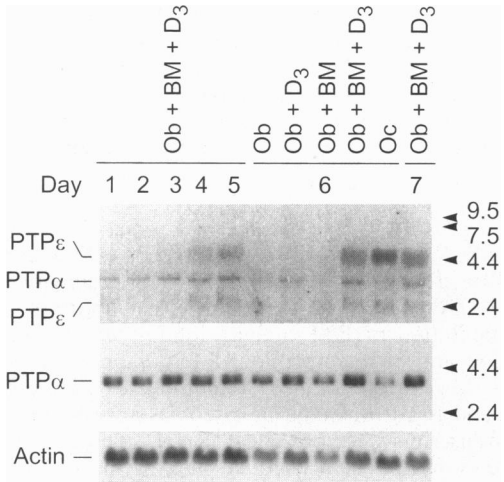

FIG. 3. Expression of PTPε and PTPα mRNA during *in vitro* osteoclast differentiation. Mouse bone marrow cells (BM) and calvaria osteoblastic cells (Ob) were cocultured in the presence of $1,25(OH)_2D_3$ (D_3) as described in text. RNA preparations and Northern hybridizations were done as described in text. Total RNA was isolated at the indicated days of coculture, and 20 μg of total RNA was used on each lane. The lane that represents RNA isolated from a 80–90% pure population of *in vitro*-generated osteoclasts (Oc) contains 10 μg of total RNA. Sizes are shown in kb.

osteoclasts and not in osteoblasts and that its expression is related to osteoclast differentiation.

Since PTPα has a widespread tissue distribution and is very similar to PTPε, we tested its expression as reference (Fig. 3). PTPα cDNA hybridized to the same filters as PTPε, showing that PTPα mRNA was constitutively expressed and did not change during osteoclast differentiation. It was present both in osteoblasts cultured alone or with bone marrow cells, as its expression did not depend on $1,25(OH)_2D_3$. The level of PTPα transcripts was not enriched in RNA isolated from purified osteoclasts.

PTPε Enzymatic Activity and Its Inhibition by ALN. To study the enzymatic activity of PTPε, we expressed the cytoplasmic domain of PTPε in bacteria as a GST-fusion protein. After purification, we tested the enzymatic activity of the GST-PTPε fusion protein with a synthetic substrate, FDP (see methods), recently found to be a very good substrate for various PTPs. PTPε effectively dephosphorylated the FDP, and the reaction was linear for at least 2 hr (Fig. 4*A*). PTPε dephosphorylated FDP with a K_m = 70 μM and a V_{max} of 6 μmol/mg per min. Similar values were obtained with PTPσ, CD45, and PTP1B (25, 26). As reported with other PTPs, the enzymatic activity of PTPε was sensitive to orthovanadate with an IC_{50} of 0.3 μM and to PAO with an IC_{50} of 18.2 μM (Fig. 4*B*). Moreover, ALN an amino-BP that inhibits bone resorption *in vivo*, inhibited PTPε with an IC_{50} of 3 μM. Etidronate, another BP, also inhibited PTPε activity with an IC_{50} of 2 μM. Similar results were obtained when the GST moiety of the GST-PTP fusion protein was cleaved with thrombin (data not shown). The inhibition by BPs was not restricted to PTPε, as it also inhibited other PTPs such as CD45 and PTPσ. ALN and etidronate inhibited the PTP CD45 with IC_{50} values of 8 μM and 2 μM, respectively. Similarly, ALN and etidronate inhibited PTPσ with similar IC_{50} values in the range of 0.5–3 μM. At concentrations that inhibited the PTPs, ALN did not inhibit the activity of other enzymes involved in phosphate metabolism. Serine/threonine phosphatase PP-1, calf intestine alkaline phosphatase, uteroferrin (acid phosphatase), yeast pyrophosphatase, and the tyrosine kinases c-*src* and v-*abl* were not inhibited at millimolar concentrations of ALN (Table 1).

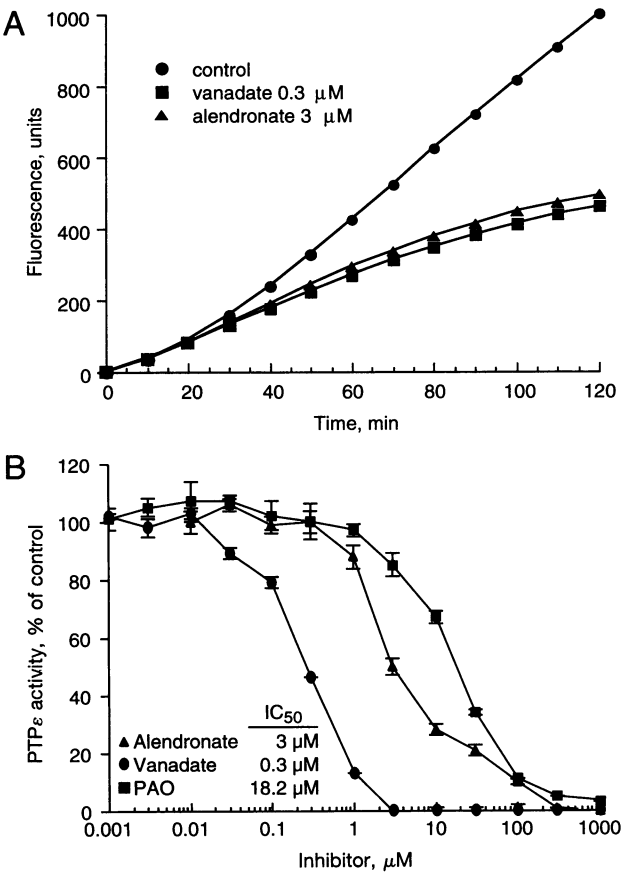

FIG. 4. Inhibition of the enzymatic activity of PTPε by ALN, orthovanadate, and PAO. The dephosphorylation assays of FDP by PTPε were performed in triplicate as described in text. (*A*) Reactions were performed at ambient temperature at a substrate concentration of 50 μM. ALN (3 μM) and vanadate (0.3 μM) were added to the reaction buffer with FDP, and the reaction was started by the addition of enzyme (0.4 μg of protein). (*B*) PTPε was incubated with inhibitor for 2 hr at ambient temperature prior to addition of substrate (50 μM). The values depict the reaction in the linear range of enzymatic activity when <10% of the substrate was used.

PTP Activity and Bone Resorption. To examine if ALN inhibition of PTP activity could be related to inhibition of bone resorption, we compared the effects of ALN, orthovanadate, and PAO on *in vitro* bone resorption by rat osteoclasts. Primary osteoclasts that were isolated from neonatal rats were placed on bone slices and incubated with vehicle or PTP inhibitors, and the number of resorption pits was determined. All three compounds markedly inhibited the number of resorption pits at concentrations above 10 μM ALN, 1 μM orthovanadate,

Table 1. Effect of alendronate on PTKs and phosphatases

Enzyme	Substrate	ALN, mM	% of control activity*
PTKs			
c-*src*	src	3	107
v-*abl*	src	1	97
Alkaline phosphatase	Tyrosine Phosphate	2	80
Ser/Thr phosphatase PP-1	KR_pTIRR	1	65
Uteroferrin (TRAP)	pNPP	1	120
Pyrophosphatase	PP_i	2	115

pNPP, *p*-nitrophenyl phosphate; KR_pTIRR, Lys-Arg-Thr(*P*)-Ile-Arg-Arg.
*Data are expressed as relative activity versus controls. The assays were performed as described in *Materials and Methods.*

and 0.1 μM PAO, respectively (Fig. 5*A*). Since we found no other enzymes that are inhibited by ALN, these findings suggest that PTP activity is essential for osteoclast mediated bone resorption.

PTP Activity and Osteoclast Differentiation. It has been previously reported that BPs inhibit osteoclast formation in culture (33). We compared the effects of the three PTP inhibitors in this *in vitro* osteoclast formation system. Cocultured mouse bone marrow cells and mouse calvaria osteoblasts were treated after 2 days with the PTP inhibitors, and the number of multinucleated TRAP$^+$ cells was determined on day 7 of coculture. ALN (30 μM), orthovanadate (10 μM), and PAO (0.1 μM), markedly inhibited the formation of multinucleated TRAP$^+$ cells (Fig. 5*B*). However, the development of mononucleated TRAP$^+$ cells seemed unaffected, suggesting that the three compounds inhibit osteoclast formation at a similar step in the maturation pathway—the fusion of mononuclear precursors. At the respective concentrations, the three PTP inhibitors had no apparent toxic effects on either osteoblasts or bone marrow cells, as assessed microscopically, in the coculture.

To test the temporal susceptibility to PTP inhibitors during osteoclast formation, we added orthovanadate at different days of coculture and monitored the number of multinucleated TRAP$^+$ cells on day 7. Addition of orthovanadate as late as day 6 for the last 24 hr inhibited the formation of multinucleated osteoclastic cells by 80%; when added earlier, the inhibition was 90% or higher (Fig. 6). These findings suggest that PTP activity is essential during a late stage in the formation of multinucleated TRAP$^+$ cells.

Inhibition of osteoclast formation by PTK inhibitors was recently reported (34, 35). We found that the PTK inhibitor geldanamycin also completely inhibited the differentiation of bone marrow cells into multinucleated TRAP$^+$ cells at 0.2 μM (data not shown). However, in contrast to PTP inhibitors, geldanamycin addition at the initiation of the coculture inhibited the development of mononuclear TRAP$^+$ cells, suggesting effects at an earlier stage of osteoclast maturation.

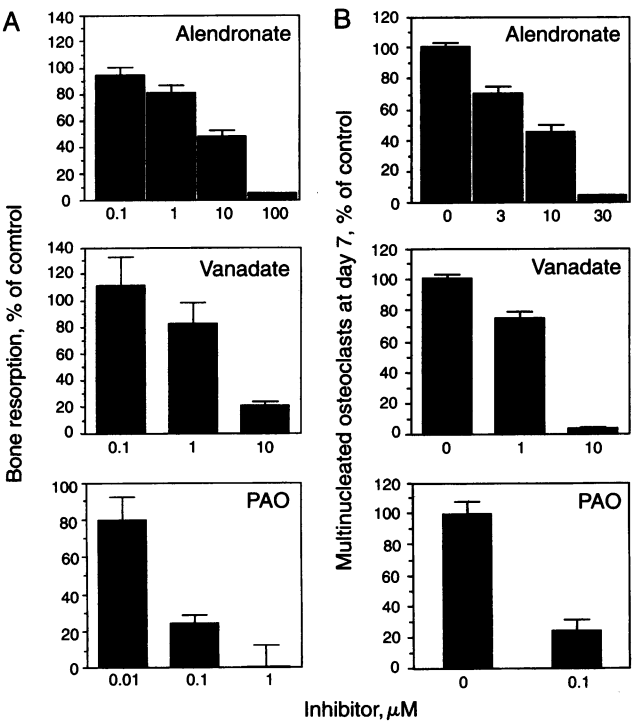

FIG. 5. Effect of PTP inhibitors on *in vitro* bone resorption and osteoclast formation. (*A*) The PTP inhibitors ALN, orthovanadate, and PAO were added at the beginning of the 24-hr bone resorption assay at the indicated concentrations under the conditions described in text. (*B*) Mouse bone marrow cells and calvaria osteoblastic cells were cocultured in the presence of 1,25(OH)$_2$D$_3$. The PTP inhibitors ALN, orthovanadate, and PAO were added at the indicated concentrations after 1 day of coculture (dilution of 1:1000 of stock solutions), and the number of TRAP$^+$ multinucleated cells was counted as described in text.

DISCUSSION

This study describes the cloning of mPTPε cDNA, expression of mPTPε in osteoclasts, and inhibition of its activity by ALN, an amino-BP used in the treatment of osteoporosis. Furthermore, inhibition of PTP activity inhibits *in vitro* osteoclast formation and bone resorption. PTPε expression correlates with *in vitro* differentiation of multinucleated mouse osteoclasts and is not detected in osteoblastic cells. In addition, PTPε transcripts were present in RNA isolated from human giant cell tumors (osteoclastoma) and in chicken osteoclasts, suggesting its presence in osteoclasts across species. The partial sequence of hPTPε was previously cloned from placenta (32) and in murine tissues. PTPε expression was shown to be restricted to testis and brain (36). Among cell lines it is expressed in IL-3-dependent myeloid leukemia cells (DA-3) but not in T cells (DA-2), colony-stimulating factor-dependent macrophages (Bac 12F5), or B cells (NFS-112 or DA-8) (36). Its expression among tissues thus seems to be relatively restricted. The presence of PTPε in osteoclasts and placenta raises the possibility that it may play a certain role in multinucleated cells. In this study, PTP inhibitors blocked specifically the formation of multinucleated osteoclasts from mononuclear precursors. The PTPs responsible for this effect and their substrates remain unknown. Since all cells express multiple PTPs, it is likely that different PTPs control osteoclast formation or bone resorption. This could explain the different dose responses for inhibition of PTPε, of bone resorption, and of osteoclast formation. Potential targets are the PTPs that modulate the activities of c-*src*-like PTKs. It was shown that c-*src* activity is regulated by tyrosine phosphorylation (11), and that PTPα or PTPD1 are associated with c-*src* (10, 37). In P19 embryonic carcinoma cells, PTPα, which is related to PTPε, increased c-*src* activity-dependent differentiation toward neuronal cells instead of mesoderm and ectoderm-like cells (38). Both PTPα and PTPε are transmembrane proteins with a short extracellular region and cytoplasmic domains that are 76% identical. PTPε may regulate the activity of the c-*src*-like PTKs in osteoclastic cells; however, direct evidence to that effect is needed.

The finding that ALN and etidronate inhibit the enzymatic activity of PTPε as well as other PTPs such as CD45 and PTPσ provides a clue and a possible molecular target for the action of BPs. Studies with radioactive ALN showed that ALN concentrates *in vivo* under osteoclasts (16). Labeled ALN was

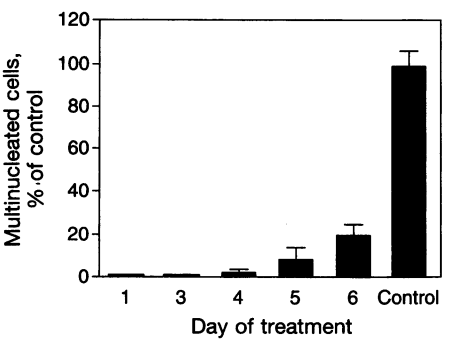

FIG. 6. Effect of orthovanadate treatment as a function of time in coculture. Orthovanadate (10 μM) was added to the *in vitro* osteoclast differentiation system at the indicated days. The number of TRAP$^+$ multinucleated cells was counted on day 7.

found in osteoclasts but not in other cell types. One possible mode of action is that ALN binds to bone mineral and is taken up by the osteoclasts that dissolve the bone mineral. Once ALN reaches critical concentrations in the cell, it inhibits osteoclast activity. Estimates indicate that at the osteoclast–bone interface, ALN concentrations can reach 100 μM or more (16), which would be enough to inhibit the PTPs expressed in osteoclasts. ALN and etidronate inhibited the PTPs examined with similar potency. The different potency observed *in vivo* may reflect differences in pharmacokinetics in cellular uptake or in the PTPs targeted *in vivo*. The same holds for the dose–response profiles of orthovanadate, PAO, and ALN, in the *in vitro* and cellular assays. Since we found no other common target for orthovanadate, PAO, and ALN except PTPs and all three suppressed both mouse osteoclast formation in culture and rat osteoclast activity in "pit" assays, PTP inhibition may relate to the mechanism of action of BPs. In osteoclast formation, the inhibitors affected the fusion of osteoclast precursors, a late step in osteoclast differentiation. Orthovanadate and PAO were active at relatively low concentrations that were not reported to influence other known targets, such as ATPase-dependent K^+ and Ca^{2+} pumps and had no cellular toxicity.

ALN inhibition of PTPs did not extend to other phosphatases, such as serine/threonine phosphatase (PP-1), calf intestine alkaline phosphatase, and TRAP, nor did ALN inhibit the c-*src* or v-*abl* PTKs. PTPs could also be BP targets in other cells, such as osteoblasts, which could modulate osteoclast activity (39) and/or respond by increasing bone formation (40). Both orthovanadate and ALN, at the concentrations used here, stimulate thymidine incorporation in quiescent osteoblasts (ref. 41, and unpublished data). The only other enzyme shown to be inhibited by BPs is squalene synthase (42).

These novel observations on BP effects on a molecular target, PTPs, raise the possibility for their involvement in the mechanism of action of these agents, consistent with the documented role of tyrosine phosphorylation in osteoclast differentiation and function.

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