Journal of Cellular Biochemistry 97:940–955 (2006)

An Osteoclastic Protein-Tyrosine Phosphatase Is a Potential Positive Regulator of the c-Src Protein-Tyrosine Kinase Activity: A Mediator of Osteoclast Activity

K.-H. William Lau,* Li-Wha Wu, Matilda H.-C. Sheng, Mehran Amoui, Sung Min Suhr, and David J. Baylink

Department of Medicine and Biochemistry, Musculoskeletal Disease Center, Jerry L. Pettis Memorial VA Medical Center, Loma Linda, California

Abstract This study tested the hypothesis that an osteoclastic protein-tyrosine phosphatase, PTP-oc, enhances osteoclast activity through c-Src activation. The effects of several resorption activators and inhibitors on PTP-oc expression, resorption activity, and c-Src activation were determined in rabbit osteoclasts. PTP-oc expression was assayed with immunoblots and semi-quantitative RT-PCR. Osteoclastic activity was determined by the resorption pit assay; and c-Src activation was monitored by P-tyr^{527} (PY527) dephosphorylation, and in vitro kinase assay. Treatment of osteoclasts with PTH, PGE_2, $1,25(OH)_2D_3$, IL-1, but not RANKL or IL-6, significantly stimulated resorption activity, increased PTP-oc mRNA and protein levels, and reduced c-Src PY527 level with corresponding activation of c-Src protein-tyrosine kinase activity. The PTP-oc antisense phosphorothioated oligo treatment blocked the basal and IL-1α-mediated, but not RANKL-mediated, resorption activity of isolated osteoclasts. The antisense oligo treatment also significantly reduced the average depth of resorption pits created by rabbit osteoclasts under basal conditions. Calcitonin and alendondrate, significantly reduced resorption activity and PTP-oc expression, and increased c-Src PY527 with corresponding reduction in its PTK activity. The cellular PTP-oc protein level correlated with the resorption activity. Among the various signaling proteins co-immunoprecipitated with PTP-oc, the resorption effectors caused corresponding changes in the tyrosyl phosphorylation level of only c-Src. The GST–PTP-oc fusion protein dephosphorylated PY-527-containing c-Src peptide in time- and dose-dependent manner in vitro. In summary, (1) PTP-oc is regulated in part at transcriptional level, (2) upregulation of PTP-oc in osteoclasts led to c-Src activation, and (3) PY527 of c-Src may be a cellular substrate of PTP-oc. These findings are consistent with the hypothesis that PTP-oc is a positive regulator of c-Src in osteoclasts. J. Cell. Biochem. 97: 940–955, 2006.

Key words: protein-tyrosine phosphatase; osteoclasts; resorption; c-Src; protein-tyrosine kinase

Bone resorption is determined by the number as well as the activity of mature osteoclasts. Recent studies of the receptor activator of NFκB ligand (RANKL) regulatory system have disclosed a great deal of information concerning the molecular mechanisms regulating osteoclast differentiation and formation. However, the regulatory mechanism(s) controlling osteoclast activity remains largely undefined. While it is likely that the molecular mechanism regulating osteoclast activity is complex and involves multiple signaling pathways (Suda et al., 1997), there is compelling evidence that c-Src (and its signaling pathway) is essential since targeted disruption of the c-Src proto-oncogene in mice resulted in a form of osteopetrosis, characterized by inactive osteoclasts and the lack of osteoclastic resorption [Soriano et al., 1991]. Abrogation of c-Src expression in osteoclasts also led to complete failure of formation of ruffled borders [Boyce et al., 1992].

The *c-Src* gene encodes a cytosolic protein-tyrosine kinase (PTK). There is evidence that the PTK activity of c-Src is important for the

Grant sponsor: National Institutes of Dental and Craniofacial Research; Grant number: RO1 DE13097; Grant sponsor: VA Merit Review Grant.

Li-Wha Wu's present address is Institute of Molecular Medicine, National Cheng Kung University, Tainan, Taiwan, Republic of China.

Sung Min Suhr's present address is Department of Biochemistry, Yonsei University, Seoul, Republic of Korea.

*Correspondence to: K.-H. William Lau, PhD, Musculoskeletal Disease Center (151), Jerry L. Pettis Memorial VA Medical Center, 11201 Benton Street, Loma Linda, CA 92357. E-mail: william.lau@med.va.gov

Received 3 May 2005; Accepted 2 September 2005

DOI 10.1002/jcb.20667

functional activity of mature osteoclasts. Active osteoclasts expressed high levels of c-Src PTK activity on ruffled border membranes [Tanake et al., 1992]. The PTK activity of c-Src correlated with the number of actively resorbing osteoclasts in vivo [Boyce et al., 1992] and with the osteoclast activity in vitro [Yoneda et al., 1994]. Specific inhibitors of the c-Src PTK activity completely abolished the bone resorption activity of treated osteoclasts [Yoneda et al., 1993; Hall et al., 1994; Blair et al., 1996; Missabach et al., 1999]. However, the contention that the PTK activity of c-Src is essential for c-Src-mediated osteoclast activation has been challenged by the findings that transgenic expression of Y416F and K295R kinase-deficient c-Src mutants in c-Src knockout mice partially rescued the osteopetrotic phenotype [Schwartzberg et al., 1997]. On the other hand, the facts that the kinase-deficient c-Src mutants, especially the Y416F mutant, still have low levels of PTK activity, and that transgenic expression of these kinase-deficient mutants in c-Src knockout mice only partially rescued the osteopetrotic phenotype suggest that the PTK activity must still have a role in the manifestation of osteoclast activity, although it might not be required.

The c-Src PTK activity is regulated primarily by its tyr^{527} phosphorylation (PY-527) status: the phosphorylation inactivates, and the dephosphorylation activates, its PTK activity [Brickell, 1992]. Thus, it may be postulated that the protein-tyrosine phosphatase (PTP) activity, responsible for the dephosphorylation of PY527 of c-Src, is a positive regulator of the PTK activity of c-Src and its signaling pathway(s) in osteoclasts and, as such, is a positive regulator of osteoclastic resorption. Consistent with this premise are the findings that $1,25(OH)_2D_3$ stimulated the c-Src PTK activity in avian osteoclast precursor cells by increasing the expression of a PTP [Chappel et al., 1997] and that resorption activators, such as PTH, activated, while inhibitors, such as calcitonin, inhibited the c-Src PTK activity in osteoclasts [Yoneda et al., 1994].

Several years ago, we have discovered an osteoclastic transmembrane PTP, termed PTP-oc [Wu et al., 1996]. PTP-oc is structurally unique in that, unlike most transmembrane PTPs, it has a very short (only eight amino acid residues) extracellular domain, lacks a signal peptide proximal to the N-terminus, contains only a single PTP catalytic domain, and is relatively small (~47 kDa). With the exception of an additional 28 amino acid residues near the N-terminus of PTP-oc, PTP-oc shows sequence identity with the intracellular domain of a renal receptor-like PTP, glomerular epithelial protein 1 (GLEPP1), which is also known as PTP-U2, PTPRO, or PTP-ϕ [Wiggins et al., 1995; Wu et al., 1996; Aguiar et al., 1999]. Although it has generally been assumed that PTP-oc is a truncated variant of GLEPP1 [Aguiar et al., 1999], we have recently shown that PTP-oc is an individual gene and that the expression of PTP-oc is driven by an alternative, intronic, tissue-specific promoter [Amoui et al., 2003]. GLEPP1 and PTP-oc are each expressed in a tissue-specific manner and each showed tissue-specific functions: GLEPP1 is primarily expressed in the kidney and the brain [Seimiya et al., 1995; Wiggins et al., 1995] and has important functions in the kidney [Wharram et al., 2000]. In contrast, PTP-oc is expressed predominantly in several hematopoietic cell types, including B lymphocytes and cells of monocyte–macrophage lineage (precursors of osteoclasts), and mature osteoclasts [Seimiya et al., 1995; Wu et al., 1996; Aguiar et al., 1999; Suhr et al., 2001]. We have demonstrated that suppression of the PTP-oc expression in rabbit osteoclasts by a PTP-oc antisense oligodeoxynucleotide (oligo) markedly reduced the basal as well as the PTH- and $1,25(OH)_2D_3$-induced bone resorption activity, and that suppression of PTP-oc expression was accompanied by an increase in PY527 level of c-Src [Suhr et al., 2001]. We recently also reported that osteoclast-like cells derived from U937 cells overexpressing wild-type PTP-oc produced larger and deeper resorption pits than osteoclast-like cells derived from control U937 cells; and conversely, osteoclast-like cells derived from U937 cells overexpressing phosphatase-dead PTP-oc yielded much smaller and shallower resorption pits than control osteoclast-like cells [Amoui et al., 2004]. Overexpression of wild-type PTP-oc also led to activation, and overexpression of phosphatase-dead PTP-oc resulted in inhibition, of the c-Src signaling pathway in these transgenic cells [Amoui et al., 2004]. Consequently, our previous findings indicate that PTP-oc is an important positive regulator of osteoclast activity, and suggest that PTP-oc could be responsible for the dephosphorylation of PY527 of c-Src and the activation of osteoclasts.

This study sought to assess the possibility that PTP-oc is a positive regulator of the c-Src PTK activity and the resorption activity of rabbit osteoclasts by testing three specific hypotheses: (1) The PTP-oc gene expression in osteoclasts would be upregulated by at least some resorption activators and downregulated by some resorption inhibitors. (2) Resorption activators that stimulate cellular PTP-oc expression would activate the c-Src PTK activity with a corresponding decrease in its PY527 level in osteoclasts. (3) The c-Src would form a tight complex with PTP-oc that is co-immunoprecipitated with the anti-PTP-oc antibody. Resorption activators should decrease, whereas resorption inhibitors should increase, the relative amounts of co-immunoprecipitated PY527 c-Src.

MATERIALS AND METHODS

Tissue culture supplies were obtained from Falcon (Oxnard, CA); Alpha-minimal Eagle's medium (α-MEM) and fetal bovine serum (FBS) were products of Life Technologies (Grand Island, NY). The enhanced chemiluminescence (ECL) detection kit was obtained from Amersham (Arlington Heights, IL). Fuji X-ray films (Fuji Co., Ltd., Tokyo, Japan) were obtained through local suppliers. Immobilon-P transfer membrane was a product of Millipore (Bedford, MA). Parathyroid hormone (PTH) (1–34) was obtained from Bachem (Torrance, CA). $1,25(OH)_2D_3$ was a product of Biomol Biomolecules Research Laboratory (Plymouth Meeting, PA). Prostaglandin E_2 (PGE_2) and calcitonin were from Sigma (St. Louis, MO). Recombinant human interleukin-1α (IL-1α), IL-6, and anti-PY-527 polyclonal antibody were products of Biosource International (Camarillo, CA). Recombinant murine soluble RANKL (sRANKL) was from PeproTech (Rocky Hill, NJ). Anti-c-Src, anti-Erk1, anti-Erk2, anti-rasGAP, anti-Shc, anti-Grb2, and anti-phosphotyrosine (anti-PY) antibodies were purchased from UpState Biotechnology (Lake Placid, NY), Santa Cruz Biotechnology (Santa Cruz, CA), or BD Transduction Laboratories (San Diego, CA). Alendronate was a generous gift of Merck (West Point, PA). All other reagents were obtained from either Sigma or Fisher (Los Angeles, CA).

Rabbit Osteoclasts Isolation

Rabbit osteoclasts were isolated from long bones of 4–7-day-old New Zealand White rabbits (Irish Farm, Norco, CA) according to Tezuka et al. [1992]. Very briefly, after removal of soft tissues, the bones were minced in α-MEM supplemented with 5% FBS. Bone cells were dissociated from bone fragments by vigorous vortexing. The unfractionated bone cells were collected by centrifugation and plated in α-MEM containing 5% FBS for 4 h. Non-adherent hematopoietic cells and loosely attached stromal cells were removed with a brief pronase E (0.001%) treatment. The resulting tartrate-resistant acid phosphatase-positive, multinucleated, osteoclasts were >90% in purity and were used immediately after isolation.

Measurement of PTP-oc mRNA Transcript Level

A semi-quantitative RT-PCR assay was used to quantify PTP-oc mRNA level. Briefly, total RNA, isolated from rabbit osteoclasts according to standard procedure [Wu et al., 1996], was treated with RNA-free DNAse I (Roche Applied Science, Indianapolis, IN), which was then heat-inactivated at 80°C for 10 min. Complimentary DNA (cDNA) was synthesized using Superscript II reverse transcriptase at 42°C for 1 h, and the cDNA product was the template in subsequent PCR amplification reactions using Vent (exo^-) DNA polymerase (New England Biolabs, Beverly, MA) with the following PTP-oc primer set: sense primer, 5′-CTC TGT CAC TTG AAC ACC TG-3′; antisense primer, 5′-TCA CAA TGC CAT CAA TGT CC-3′. The PCR reaction was subjected to a hot start for 3 min at 94°C, followed by 26 amplification cycles: 45 s at 94°C, 45 s at 60°C, and 2 min at 74°C. The PCR products were analyzed on 1.3% agarose gel, and the 319-bp PTP-oc PCR fragment was probed with the [^{32}P]labeled PTP-oc antisense 20-oligomer (5′-CTC CTT GGT AAT CCA TAG AG-3′). Each cDNA sample was also PCR amplified for 24 cycles for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript (as the control for cDNA loading) using a commercial GAPDH amplifer kit (Clontech Laboratories, Inc., Palo Alto, CA). The 452-bp GAPDH PCR product was probed with the [^{32}P]labeled antisense oligo (5′-CAC GGA AGG CCA TGC CAG TGA GCT TCC CG-3′). The relative level of PTP-oc transcript (normalized against the GAPDH transcript) is reported as percentage of respective vehicle-treated control. Each RT-PCR assay was performed in triplicate and repeated twice.

PTP-oc Antisense Oligodeoxynucleotide Studies

The design of the 20-mer PTP-oc antisense phosphorothioated oligo (5′-GsTsAs AsCsCs AsTsTs GsTsTs GsAsGs AsCsAs GsC-3′) and the corresponding scramble oligo (5′-GsTsCs TsAsGs TsTsAs GsCsAs CsGsAs AsTsGs CsA-3′) was described previously [Suhr et al., 2001] and was custom-synthesized, HPLC-purified by Genset Corp. (La Jolla, CA). Briefly, isolated rabbit osteoclasts were plated on dentine discs in DMEM supplemented with 10% fetal bovine serum for 3 days in the absence or presence of 1 μM PTP-oc antisense or scramble oligo. After the pre-treatment, the osteoclasts on dentine discs were cultured for 3 additional days in DMEM (without serum) in the presence of each test oligo (with or without 100 ng/ml sRANKL or 10 ng/ml IL-1α) to induce resorption pit formation. After the treatment, each dentine disc was stained for TRAP activity, and osteoclasts on the disc were removed by sonication and the resorption area per resorption lacunae was then determined as described previously.

Resorption Pit Formation Assay

The bone resorption activity of osteoclasts was measured with the resorption pit formation assay on dentine discs as previously described [Suhr et al., 2001]. The average resorption activity per osteoclast (an index of mean osteoclast activity) was determined by dividing total resorption lacunae area by the number of resorption pits.

The average resorption pit depth was determined by confocal microscopy using an Olympus IX70 inverted confocal microscope workstation (Olympus, Melville, NY) as described previously [Amoui et al., 2004]. Briefly, after the resorption pit assay under basal conditions, the acid hematoxylin-stained pits on the dentin disc were mounted on a glass slide in 50% glycerin (v/v). The coverslip was sealed, dried, and mounted upside down on the microscope. The resorption pits were viewed on the Olympus microscope fitted with a confocal argon–krypton mixed gas laser. Serial images of the pits at 1-μm intervals from the top surface of the dentin disc to the bottom of the pit were obtained using a z-plane motorized substage. The average depth was calculated from the number of 1-μm slices needed to reach the bottom of the pit from the top of the disc. At least 20 pits per disc and three replicate discs per group were analyzed.

Measurement of Cellular PTP-oc Protein Levels

The cellular PTP-oc level in rabbit osteoclasts was determined using a guinea pig polyclonal antibody against the unique region of the N-terminus of rabbit PTP-oc [Wu et al., 1996] on Western immunoblots as described previously [Suhr et al., 2001].

Measurement of c-Src PY527 Levels

The c-Src PY527 level was measured using a phospho-specific polyclonal antibody against PY527 of c-Src on Western immunoblots as described previously [Suhr et al., 2001].

In Vitro c-Src PTK Activity Assay

The c-Src PTK activity was measured with a synthetic peptide substrate. Briefly, osteoclasts were extracted with the radioimmunoprecipitation assay (RIPA) buffer [50 mM of Tris-HCl, pH 8.0, 1% (v/v) of NP-40, 20 mM of EDTA, 0.1 mM of Na_3VO_4, 0.5% of sodium deoxycholate, 0.1% of sodium dodecyl sulfate (SDS), 0.05 mM of leupeptin, 1 mM of phenylmethylsulfonylfluoride, and 10 μg/ml of aprotinin]. The c-Src protein in each extract was isolated by immunoprecipitation using a monoclonal anti-c-Src antibody by standard immunoprecipitation protocol [Argetsinger et al., 1993]. The immunoprecipitate was resuspended in the PTK assay buffer (10 mM of Tris-HCl, pH 7.4, 0.1 mM of EDTA, 0.01% (v/v) of Triton X-100, 150 mM of NaCl, 10 mM of $MnCl_2$, and 0.5 mM of dithiothreitol), 5 μg/ml of Raytide (a synthetic c-Src substrate peptide) was added to the assay mixture, and the phosphorylation reaction was initiated by the addition of γ-[^{32}P]ATP (15 μM) and carried out at 37°C for 30 min. After the reaction, cellular proteins, but not the labeled Raytide, were removed by ice-cold trichloroacetic acid precipitation, and the [^{32}P]ATP and [^{32}P]PO_4 were removed with a small Dowex AG8 column. The unadsorbed labeled Raytide was spotted onto a Whatman P81 phosphocellulose paper square. After extensive washing with ice-cold 150 mM phosphoric acid, the [^{32}P]PO_4 incorporation in Raytide was determined by scintillation counting and the c-Src PTK activity was calculated and normalized against cellular protein.

PTP-oc Co-Immunoprecipitation

Briefly, osteoclasts were lysed in RIPA and the cell extract was immunoprecipitated with anti-PTP-oc or an antibody against the signaling protein-of-interest. The immune complex were then resolved on 10% SDS–PAGE. The tyrosyl-phosphorylated (PY) proteins, PTP-oc, and signaling proteins-of-interest, were identified with Western blots.

Measurement of PY Level of Co-Immunoprecipitated Proteins

The PY level of cellular proteins co-immunoprecipitated with PTP-oc was measured as follows: an aliquot of osteoclast extract (50 μg protein) was immunoprecipitated with the affinity-purified anti-PTP-oc antibody. The immune complex was resolved on 10% SDS–PAGE. The PY level of each protein-of-interest (i.e., c-Src, Erk1, Erk2, rasGAP, Shc, and Grb2) was measured by Western immunoblotting using a commercial anti-PY monoclonal antibody, followed by ECL and quantitation by laser densitometry. The blot was then stripped and reblotted with an antibody specific for each protein-of-interest. The PY level of each signaling protein-of-interest was normalized against the level of respective total protein-of-interest.

PY-527 c-Src Phosphatase Assay

The PY-527-containing c-Src (521–533) peptide [TSTEPQY(p)QPGENL] was purchased from MP Biomedicals, LLC (Aurora, OH). Glutathione S-transferase (GST)–PTP-oc fusion protein was prepared and isolated as described previously [Wu et al., 1996]. The dephosphorylation of PY-527-containing c-Src peptide by PTP-oc was determined with a malachite green microtiter plate assay [Harder et al., 1994], using a commercial PiBlue™ phosphate assay kit (BioAssay systems, Hayward, CA). The dephosphorylation reaction was initiated by adding an aliquot (10 μl) of purified GST–PTP-oc fusion protein in a final volume of 50 μl of reaction mixture including 25 mM HEPES (pH 7.0), 5 mM EDTA, 50 mM NaCl, 10 mM DTT, and 0.5 mM PY-527-containing c-Src peptide in a 96-well microtiter plate. The reaction was carried out at 30°C for the indicated length of time and was terminated by addition of 100 μl PiBlue™ malachite green reagent. The mixture was then mixed and incubated at 30°C at room temperature for 30 min for color development, and the absorbance at 660 nm was determined in a 96-well plate reader. Values for the amount of Pi release from the PY-527-containing c-Src peptide were obtained from comparison with a standard curve.

Statistical Analysis

Results are shown as mean ± SEM of 3–6 replicates or repeat measurements unless otherwise indicated. Statistical significance was determined with two-tailed Student's *t*-test and the difference was considered significant when $P < 0.05$.

RESULTS

Regulation of PTP-oc Expression by Resorption Effectors in Rabbit Osteoclasts

We have previously shown that a 24-h treatment with an effective dose of PTH or 1,25 $(OH)_2D_3$ each significantly increased cellular PTP-oc protein level in rabbit osteoclasts [Suhr et al., 2001]. In this study, a 24-h treatment with recombinant human IL-1α at 10 ng/ml also significantly ($P < 0.01$) increased cellular PTP-oc protein level by ~twofold in rabbit osteoclasts (Fig. 1). However, recombinant murine sRANKL, at a dose as high as 100 ng/ml, did not alter the cellular PTP-oc protein level. Recombinant human IL-6 at 50 ng/ml slightly increased cellular PTP-oc protein levels (by 25–50%), but the increase did not reach statistically significant level. The murine sRANKL and human IL-6 were active, since they each showed significant effects in the assay of the differentiation of RAW264.7 cells into osteoclast-like cells (data not shown). Alendronate and calcitonin (two resorption inhibitors) each at effective doses significantly decreased cellular PTP-oc protein levels up to 30% (data not shown).

To confirm that these resorption effectors affect PTP-oc gene expression, we measured the PTP-oc mRNA level in rabbit osteoclasts by semi-quantitative RT-PCR after a 24-h treatment with PTH (10 μM), $1,25(OH)_2D_3$ (10 nM), PGE_2 (1 μM), sRANKL (100 ng/ml), and alendronate (10 nM). The test resorption activators (PTH, $1,25(OH)_2D_3$, and PGE_2) each significantly increased the PTP-oc mRNA transcript level (normalized against GAPDH) by two- to threefold, while the test resorption inhibitor (alendronate) significantly decreased it by 25% (Fig. 2). sRANKL at the test dose had no significant effect.

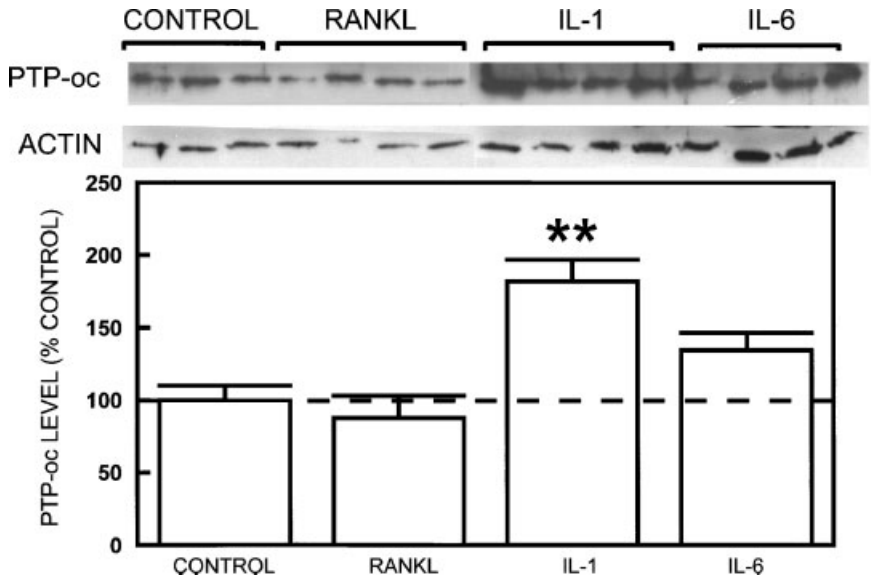

Fig. 1. Effects of sRANKL, IL-1α, and IL-6 on the cellular PTP-oc protein level in rabbit osteoclasts. Freshly isolated rabbit osteoclasts were treated with 100 ng/ml of recombinant murine sRANKL, 10 ng/ml of recombinant human IL-1α, 50 ng/ml of recombinant human IL-6, or solvent control, respectively, for 24 h. The cellular PTP-oc level was measured with Western blot normalized against cellular actin level. **Top panel** shows the Western blot and **bottom panel** summarizes the results of the cellular PTP-oc level normalized against the actin level. The results are shown as percentage of the vehicle control (mean ± SEM of triplicate). The dashed line indicates the 100% of the vehicle control. $^{**}P < 0.01$.

To evaluate the relationship between PTP-oc expression and osteoclast activity, we performed correlation analysis between the effect of the test resorption activators (with the exception of sRANKL and IL-6) and inhibitors on osteoclast activity (measured by pit formation assay) and their effects on cellular PTP-oc level. There was a significant ($P < 0.01$) positive correlation ($r = 0.985$), supporting our previous conclusion that PTP-oc is a positive regulator of osteoclast activity [Suhr et al., 2001]. IL-6 and sRANKL were not included in the analysis because these two agents did not significantly affect the cellular PTP-oc level in rabbit osteoclasts.

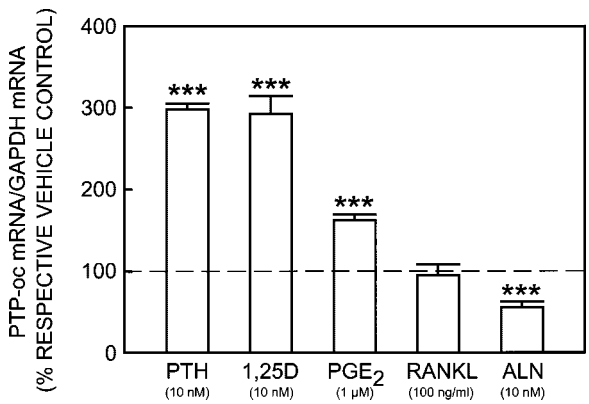

Fig. 2. Effect of bone resorption effectors on the PTP-oc mRNA transcript level in rabbit osteoclasts. Freshly isolated rabbit osteoclasts were treated with 10 nM of PTH, 10 nM of $1,25(OH)_2D_3$ (1,25D), 1 μM of PGE_2, 100 ng/ml of recombinant murine sRANKL, or 10 nM of alendronate (ALN), and their corresponding solvent vehicle for 24 h. The PTP-oc mRNA transcript level was determined with a semi-quantitative RT-PCR assay and normalized against the GAPDH mRNA transcript level. Results are shown as mean ± SEM of at least three replicate analyses. The dashed line indicates the 100% of the respective vehicle control. $^{***}P < 0.001$.

Effects of PTP-oc Antisense Oligodeoxynucleotide on the Bone Resorption Activity of Isolated Rabbit Osteoclasts

We have previously shown that suppression of the PTP-oc expression by a PTP-oc antisense oligodeoxynucleotide in rabbit osteoclasts markedly reduced the basal as well as the PTH- and $1,25(OH)_2D_3$-induced bone resorption activity [Suhr et al., 2001]. Thus, we tested whether suppression of PTP-oc expression by the same antisense oligodeoxynucleotide would also affect the IL-1α- and RANKL-mediated bone resorption activity of isolated rabbit osteoclasts. We have previously shown that under our experimental conditions, this PTP-oc antisense oligo decreased cellular PTP-oc protein level by ~70% [Suhr et al., 2001]. Under our experimental conditions, IL-1α (10 ng/ml) and sRANKL (100 ng/ml) each significantly enhanced the resorption

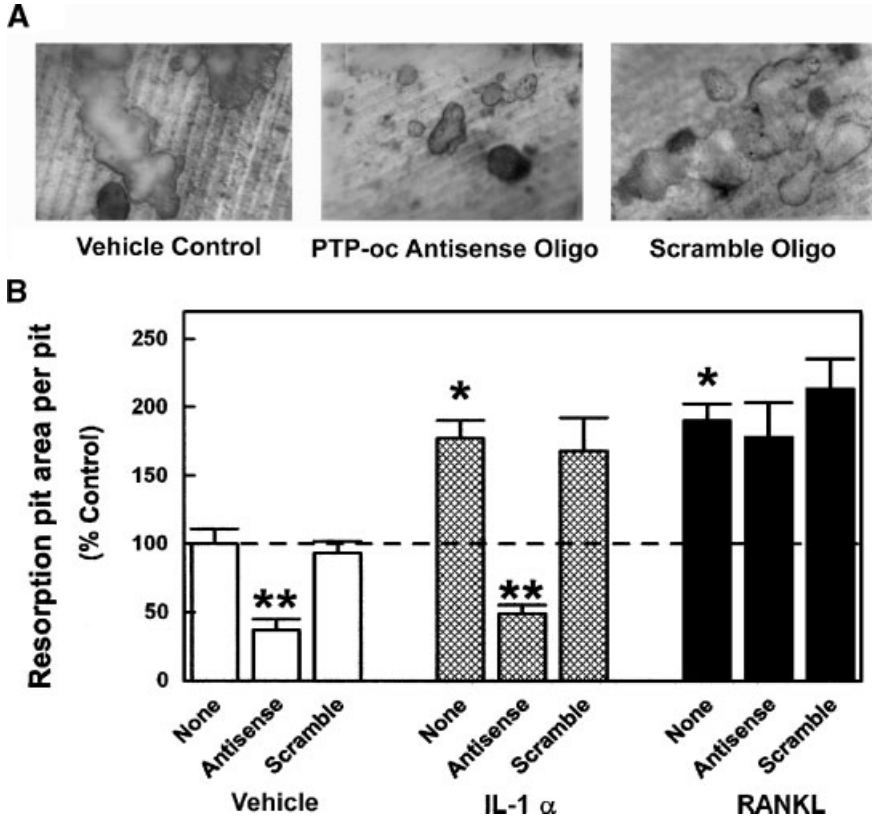

Fig. 3. Effects of PTP-oc antisense oligo on basal, IL-1α- and RANKL-induced bone resorption activity of isolated rabbit osteoclasts. The isolated rabbit osteoclasts were plated on each dentine disc and were pretreated for 3 days with PTP-oc antisense oligo, scramble oligo, or the vehicle control, respectively, and then for 3 additional days in the absence (basal) or presence of 10 ng/ml IL-1α or 100 ng/ml sRANKL, respectively. Cell medium containing respective effector and oligo was changed every 3 days. The resorption pit formation was performed as described in Materials and Methods. **Panel A** shows a representative photomicrograph of resorption pits formed by isolated rabbit osteoclasts, which were treated for 6 days with solvent vehicle (**left**), 1 μM of phosphorothioated PTP-oc antisense oligo (**middle**), or 1 μM of phosphorothioated scramble oligo (**right**). **Panel B** summarizes the results of each treatment on the resorption pit area/pit. The results are shown as mean ± SEM ($n = 6–8$ for each). $^{*}P < 0.05$ compared with the no-treatment vehicle-treated control; $^{**}P < 0.05$ compared with respective vehicle-treated control.

activity by ~ twofold (Fig. 3). The PTP-oc antisense oligo treatment significantly reduced basal and IL-1α-induced bone resorption activity by 50–60%. However, the PTP-oc antisense oligo treatment had no significant effect on the RANKL-mediated bone resorption activity. The scramble oligo did not affect basal or IL-1α- and RANKL-induced bone resorption.

Resorption activity is determined by not only the size but also the depth of resorption pits. The pit size, but not depth, of resorption pits is also determined by the migration ability of osteoclasts on the dentin disc. We have previously reported that osteoclast-like cells derived from U937 cells stably overexpressing wild-type PTP-oc created not only bigger but also significantly deeper resorption pits on dentine discs than osteoclast-like cells derived from wild-type U937 control cells; and conversely, osteoclast-like cells derived from U937 cells expressing a phosphatase-dead PTP-oc mutant created significantly smaller and shallower resorption pits than the control osteoclast-like cells [Amoui et al., 2004]. This suggests that the PTP-oc activity affects both the migration and resorption activity of osteoclast-like cells. Accordingly, we next assessed whether the PTP-oc antisense oligo treatment would also affect the resorption pit depth of bona fide rabbit osteoclasts under basal conditions. Figure 4 shows that the PTP-oc antisense oligo treatment significantly reduced the average resorption pit depth by ~60% compared to the untreated control, while the scramble oligo treatment only slightly but not significantly reduced the average resorption pit depth. These findings indicate that the

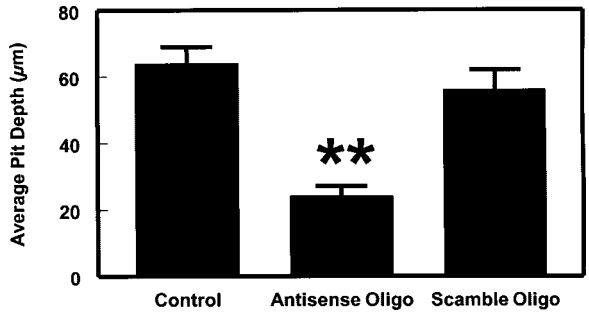

Fig. 4. Effect of PTP-oc antisense oligo on the average depth of resorption pits created by rabbit osteoclasts under basal conditions. The isolated rabbit osteoclasts were plated on each dentine disc and were pretreated for 6 days with PTP-oc antisense oligo, scramble oligo, or the vehicle control, respectively. Cell medium containing respective oligo was changed every 3 days. The average depth of resorption pits was determined by confocal microscopy as described in Materials and Methods. Results are shown as mean ± SEM of at least 20 resorption pits on each of the three replicate dentine dics per group. $^{**}P < 0.01$.

PTP-oc activity has a regulatory role in both the migration and resorption activity of rabbit osteoclasts, at least under basal conditions.

Resorption Effectors Regulated the PTK Activity and the PY527 Level of c-Src in Rabbit Osteoclasts

We next evaluated whether a 24-h treatment of PTH, $1,25(OH)_2D_3$, PGE_2, calcitonin, and alendronate would affect the PTK activity of c-Src in rabbit osteoclasts using an in vitro PTK assay. The left panel of Figure 5 shows that PTH, $1,25(OH)_2D_3$, and PGE_2 each significantly enhanced the PTK activity of c-Src; while alendronate or calcitonin each significantly reduced the c-Src PTK activity (right panel of Fig. 5). These findings are consistent with the previous findings that PTH [Yoneda et al., 1994] and $1,25(OH)_2D_3$ [Chappel et al., 1997] activated and calcitonin [Yoneda et al., 1994] reduced the c-Src PTK activity in osteoclasts, and suggest that upregulation of PTP-oc expression leads to an activation of the PTK activity of c-Src and downregulation of PTP-oc expression results in a reduction of the c-Src PTK activity in osteoclasts.

Figure 6 shows that PTH, $1,25(OH)_2D_3$, PGE_2, or IL-1α, but not sRANKL, each led to a significant reduction in c-Src PY527 level, while alendronate significantly increased c-Src PY527 level. None of the test effectors has a significant effect on total c-Src level (i.e., total c-Src/actin) (data not shown). Thus, the observed changes in the c-Src PTK activity in response to the test effectors were due to the corresponding changes in c-Src PY527 dephosphorylation.

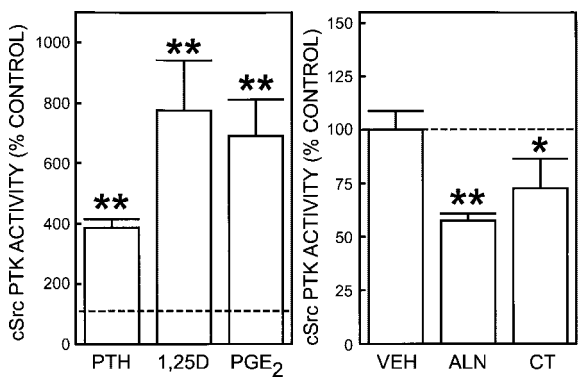

Fig. 5. Effect of resorption activators (**left panel**) and inhibitors (**right panel**) on the c-Src PTK activity in rabbit osteoclasts. Freshly isolated rabbit osteoclasts were treated with 10 nM of PTH, 10 nM of $1,25(OH)_2D_3$ (1,25D), 1 μM of PGE_2, 10 nM of alendronate (ALN), 30 nM of calcitonin (CT), or respective solvent control for 24 h. The c-Src PTK activity in each cell extract (50 μg of protein) after respective treatment was immunoprecipitated with the anti-c-Src antibody, and the PTK activity of each immunoprecipitate was assayed with the in vitro PTK assay using Raytide as the substrate. Results were shown as mean ± SEM (n = 3) of the percentage of respective solvent control. The dashed line in each panel indicates the 100% of the respective solvent control. $^{*}P < 0.05$; $^{**}P < 0.01$.

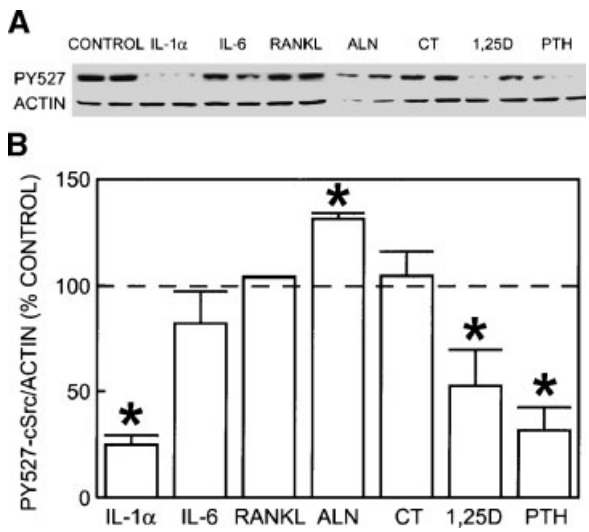

Fig. 6. Effect of resorption effectors on c-Src PY527 level in rabbit osteoclasts. Freshly isolated rabbit osteoclasts were treated with 10 ng/ml of IL-1α, 50 ng/ml of IL-6, 100 ng/ml of soluble RANKL, 10 nM of alendronate (ALN), 30 nM of calcitonin (CT), 10 nM of $1,25(OH)_2D_3$ (1,25D), 10 nM of PTH, or solvent control, respectively, for 24 h. The cellular c-Src PY527 level was measured with Western blot normalized against actin. **Panel A** shows the Western blot of PY527 levels after treatment with the indicated effector (in duplicate) and **panel B** summarizes the results of the cellular c-Src PY257 level normalized against actin. The results are shown as percentage of the vehicle control (mean ± SEM, n = 2). The dashed line indicates the 100% of the control. $^{*}P < 0.05$.

PTP-oc Forms Tight Complex With c-Src in Rabbit Osteoclasts

The co-immunoprecipitation approach was employed to identify the cellular proteins that are associated tightly with PTP-oc, using a highly specific, affinity-purified, polyclonal anti-rabbit PTP-oc antibody. There were at least 6 PY proteins co-immunoprecipitated with PTP-oc, but no significant amounts of PY proteins were immunoprecipitated with normal guinea pig IgG or non-immune serum (data not shown). The apparent M_r of the co-immunoprecipitated PY proteins was similar to that of several signaling proteins of the c-Src signaling pathway [Migliaccio et al., 1998]. These are $pp60^{c\text{-}Src}$, $pp120^{rasGAP}$, $pp44^{Erk1}$, $pp42^{Erk2}$, $pp66^{Shc}$, $pp52^{Shc}$, $pp46^{Shc}$, and $pp25^{Grb2}$. The overall PY level of many of these cellular proteins was elevated by the 24-h treatment with $1,25(OH)_2D_3$, PTH, or PGE_2, and reduced after the calcitonin or alendronate treatment.

The identity of some of the co-immunoprecipitated PY proteins was confirmed to be c-Src, rasGAP, Erk1, Erk2, Shc, and Grb2, with Western blots. This conclusion was based on findings that specific antibody against these proteins reacted strongly with a protein band with an apparent M_r, corresponding to that of each respective signaling protein. As an example, the left panel of Figure 7 shows the immunoblot of the anti-PTP-oc co-immunoprecipitated proteins with a mixture of anti-c-Src, anti-Erk1, and anti-Grb2 antibodies. The association between these signaling proteins and PTP-oc was confirmed with reverse immunoprecipitation, in which PTP-oc was first co-immunoprecipitated with each protein-of-interest and the presence of PTP-oc was verified with the anti-PTP-oc antibody (the right panel of Fig. 7). In each case, there was a single, strongly reactive, protein band corresponding to the apparent M_r of PTP-oc.

If c-Src (and not other co-immunoprecipitated PY proteins) is a substrate of PTP-oc, the resorption effectors should affect the amounts of immunoprecipitated PY c-Src (and not that of other co-immunoprecipitated PY proteins). Thus, we assessed the effects of the test resorption effectors on the PY and total levels of immunoprecipitated c-Src as well as those of other immunoprecipitated signaling proteins. Because the test effectors also have an effect on the PTP-oc protein level, the relative level of each signaling protein was normalized against PTP-oc protein level. Among the test proteins, only the relative level of total c-Src (per PTP-oc level), but not that of the other signaling proteins, was reduced by the 24-h treatment with PGE_2, PTH, or $1,25(OH)_2D_3$ (data not shown). The level of immunoprecipitated total c-Src (adjusted for PTP-oc level) was increased (three- to fivefold) by calcitonin and alendronate (data not shown).

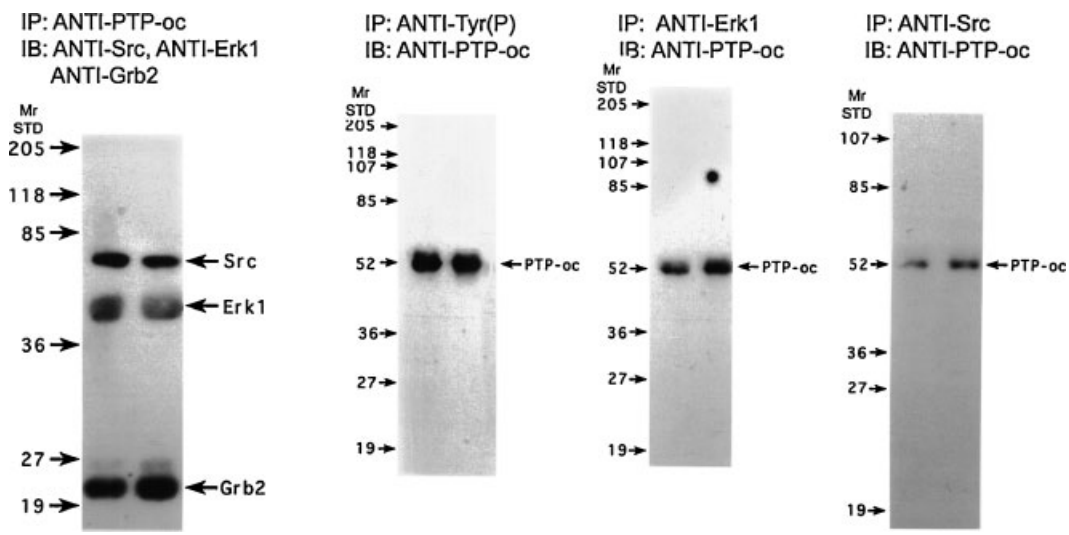

Fig. 7. Co-immunoprecipitation of signaling proteins with PTP-oc. **Left panel**: The lysate (100 μg protein) of freshly isolated rabbit osteoclast was immunoprecipitated (IP) with the anti-PTP-oc antibody. The signaling proteins were identified by immunoblotting (IB) with a mixture of specific antibodies against c-Src, Erk1, and Grb2. **Right panel**: Freshly prepared lysates (100 μg of protein) of rabbit osteoclast were first immunoprecipitated with anti-PY antibody, anti-Erk1 antibody, or anti-c-Src-antibody, respectively. The immunoprecipitated proteins from each treatment were then immunoblotted with the anti-PTP-oc antibody.

To analyze the effects of the test resorption agents on the PY level of the co-immunoprecipitated proteins, the level of the PY species of each co-immunoprecipitated signaling protein (normalized against total level) was assessed with the immunoblot assay using an anti-PY antibody. PGE_2, PTH, and $1,25(OH)_2D_3$, each markedly reduced the overall PY level of the co-immunoprecipitated c-Src. Calcitonin and alendronate each significantly increased the PY level of c-Src by $\sim$ threefold of the vehicle-treated control. These effectors had no significant effect on the PY of other test signaling proteins (Erk1, Erk2, Shc, and Grb2). Figure 8 shows an example of PGE_2 and calcitonin on the PY level of c-Src and Erk1.

To further test whether PY-527 of c-Src is a substrate of PTP-oc, we performed an in vitro dephosphorylation reaction of a commercially synthesized PY-527-containing c-Src peptide with a GST–PTP-oc fusion protein. Figure 9 shows that the GST–PTP-oc fusion protein was able to dephosphorylate the PY-527-containing c-Src peptide in a time- (panel A) and dose-dependent (panel B) manner. These findings are consistent with the premise that PY-527 of c-Src is a potential cellular substrate of PTP-oc.

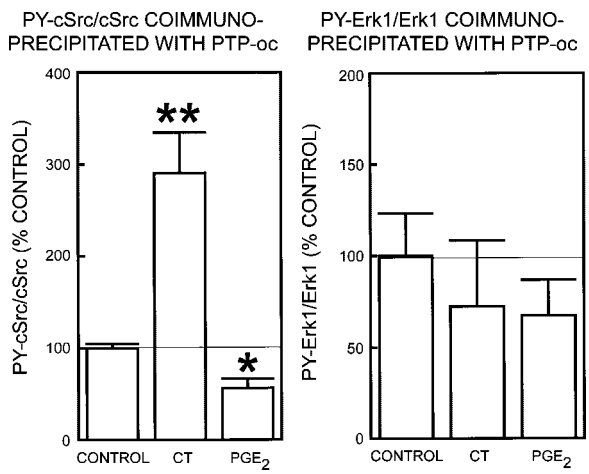

Fig. 8. Effect of resorption effectors on the overall PY level of c-Src (**left panel**) and that of Erk1 (**right panel**). Freshly isolated rabbit osteoclasts, after a 24-h with either 30 nM of calcitonin (CT) or 1 μM of PGE_2, were extracted with RIPA buffer. An aliquot (20 μg protein) of each extract was immunoprecipitated with the anti-PTP-oc antibody. The immune complex was then resolved on a 10% SDS–PAGE, and the co-immunoprecipitated c-Src and Erk1 were identified on Western blots with anti-c-Src antibody or anti-Erk1 antibody, respectively. The relative level of co-immunoprecipitated c-Src or Erk1 was determined by laser densitometry. The Western blots were then stripped and reblotted with the anti-PY antibody to determine the relative level of PY-cSrc or PY-Erk1, respectively. The relative PY-cSrc and PY-Erk1 levels were normalized against total cSrc and total Erk1, respectively. Results are shown as mean ± SEM of three replicate experiments. The thin line indicates the 100% of the vehicle control. * $P < 0.05$; ** $P < 0.01$.

DISCUSSION

The findings of our previous studies with the PTP-oc antisense oligo in rabbit osteoclasts [Suhr et al., 2001] and with osteoclast-like cells derived from U937 cells overexpressing either the wild-type or phosphatase-dead PTP-oc [Amoui et al., 2004] led to our postulation that PTP-oc plays an important role in the regulation of osteoclast activity. Accordingly, we have advanced a model of the molecular mechanism for the enhancing action of PTP-oc on osteoclast activity (Fig. 10). We postulate that at least some of the resorption activators, such as PTH, $1,25(OH)_2D_3$, PGE_2, and IL-1α, (but not RANKL or IL-6) act directly on osteoclasts to stimulate the PTP-oc gene transcription. The PTP-oc mRNA is translated into mature PTP-oc protein and incorporated into cellular membrane. The transmembrane PTP-oc dephosphorylates the PY527 residues of c-Src, which results in activation of the c-Src PTK activity and its associated signaling pathways. This in turn leads to the increase in osteoclastic activity through activation of the c-Src signaling pathway by dephosphorylating PY527 of c-Src [Suhr et al., 2001].

This report presents strong, albeit circumstantial, evidence for three aspects of this model. First, our findings that several resorption activators, for example, PTH, $1,25(OH)_2D_3$, PGE_2, and IL-1α, each, at a concentration that stimulated the resorption activity of osteoclasts in vitro, significantly increased the PTP-oc expression and that two potent resorption inhibitors (calcitonin and alendronate) each at a concentration that inhibited the resorption activity of rabbit osteoblasts in vitro, also suppressed PTP-oc expression are entirely consistent with the premise that at least some resorption effectors regulate PTP-oc expression in osteoclasts. The positive correlation between cellular PTP-oc level and resorption activity of rabbit osteoclasts after 24-h treatment with some test resorption effectors provides strong, associative evidence for a regulatory role of transcriptional control of PTP-oc gene expression in osteoclast activity. Moreover, PTP-oc antisense oligo treatment markedly reduced

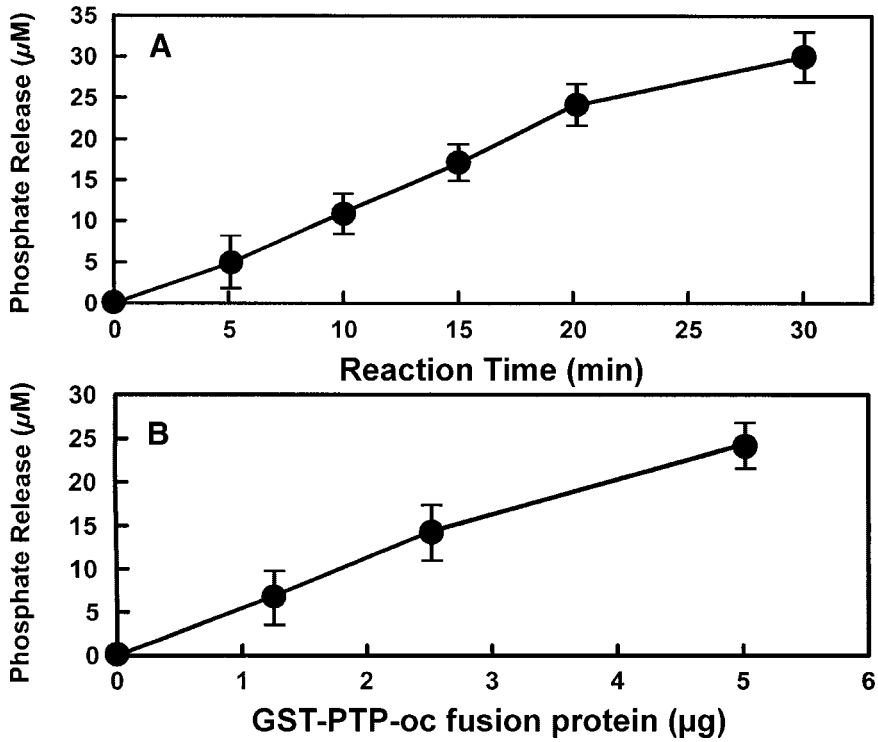

Fig. 9. Dephosphorylation of PY-527-containing c-Src peptide by GST–PTP-oc fusion protein. The dephosphorylation was carried out as described in Materials and Methods. **Panel A** shows the dephosphorylation rate as a function of time. **Panel B** shows it as a function of the amount of the fusion protein. Results are shown as mean ± SEM of three replicate.

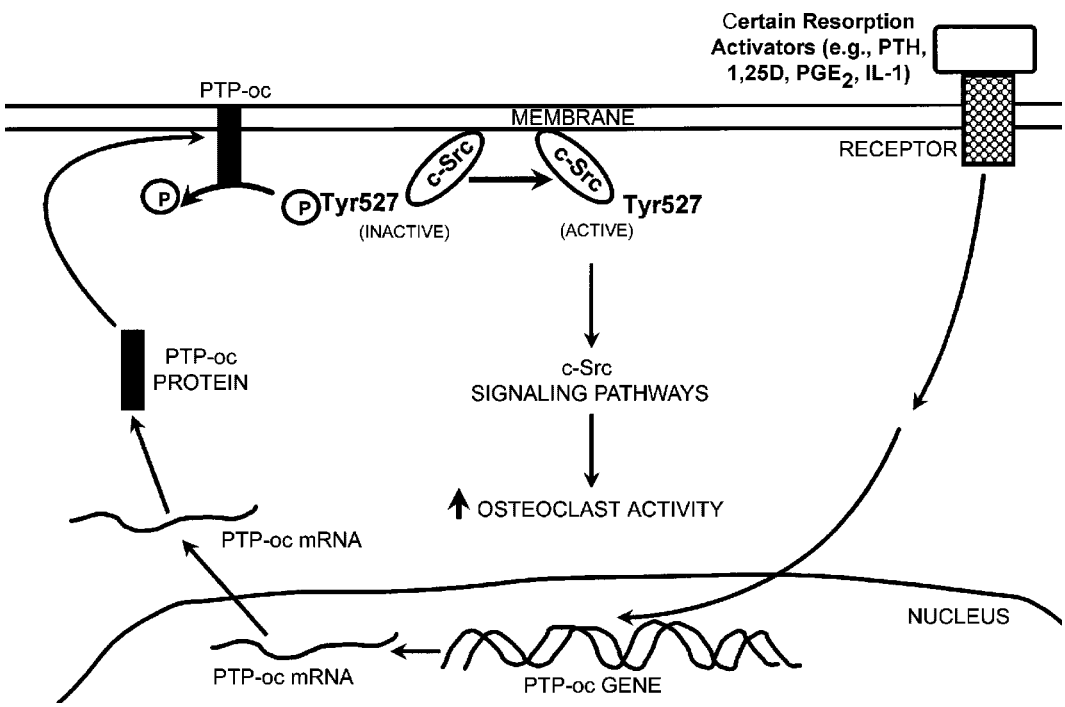

Fig. 10. A proposed model of the PTP-oc-dependent regulation of osteoclast activity. It is postulated that certain resorption activators (such as PTH, $1,25(OH)_2D_3$, PGE_2, and IL-1, but not RANKL or IL-6) bind directly on their respective receptor to stimulate the transcription of *PTP-oc* gene. The PTP-oc mRNA is then translated into mature PTP-oc, which is in turn incorporated into the cell membrane of osteoclasts, where it dephosphorylates the PY527 of c-Src. Upon PY527 dephosphorylation, c-Src PTK is activated, which then stimulates osteoclast activity through activation of the associated c-Src signaling pathway.

basal osteoclastic resorption and the resorption induced by IL-1α (which upregulated PTP-oc expression in osteoclasts) but not that by RANKL (which had no effect on PTP-oc expression). These findings support an important contention of our hypothesis that the PTP-oc expression level has a regulatory role in the osteoclast activity.

Two findings with respect to PTP-oc gene expression and regulation of rabbit osteoclast activity are noteworthy. First, PTP-oc expression was not upregulated by all resorption activators. Accordingly, soluble RANKL, a potent stimulator of osteoclast differentiation and activity [Shiotani et al., 2002], stimulated resorption activity but had no significant effect on cellular PTP-oc level. Similarly, IL-6, which stimulated osteoclast activity in part through the RANKL regulatory system [Palmqvist et al., 2002], also did not consistently upregulate PTP-oc expression. These findings led us to tentatively conclude that PTP-oc regulates osteoclast activity in a RANKL-independent manner. However, our findings do not exclude the possibility that the PTP-oc signaling pathway may still interact or crosstalk with the signaling pathway of RANKL to enhance osteoclast activity.

The second intriguing finding is the apparent direct stimulatory action of PTH on PTP-oc gene expression and bone resorption activity of rabbit osteoclasts. It has generally been accepted that mature osteoclasts lack functional PTH receptors and that the stimulatory effect of PTH on osteoclastic resorption is mediated indirectly through upregulation of the RANKL regulatory system in osteoblastic cells [Suda et al., 1999]. Our osteoclast preparations could have up to 10% contamination of marrow stromal cells. Because PTH (as well as $1,25(OH)_2D_3$ and PGE_2) could act through stromal cells to activate osteoclast formation and activity through upregulation of the RANKL system [Suda et al., 1999], we cannot rule out the possibility that the observed stimulatory effect of PTH (and/or some of the effectors, such as $1,25(OH)_2D_3$ and PGE_2) was mediated indirectly through the contaminated stromal cells. However, contrary to the general assumption that osteoclasts lack functional PTH receptor, there are several recent cytochemical demonstrations of the existence of nuclear PTH/PTHrp receptor in osteoclasts of several species [Langub et al., 2001; Faucheux et al., 2002; Gay et al., 2003; Nakashima et al., 2003]. We have also preliminary RT-PCR evidence that rabbit osteoclasts and RAW264.7-derived osteoclasts express abundant PTH receptor mRNA transcripts (unpublished observations). Similarly, mature osteoclasts have been shown to express receptors for $1,25(OH)_2D_3$ [Langub et al., 2000] and PGE_2 [Sarrazin et al., 2004]. The fact that osteoclasts express receptors for PTH, $1,25(OH)_2D_3$, and PGE_2 supports a potential direct action of these effectors on osteoclasts. Regardless of whether these effectors act directly on osteoclasts or indirectly through stromal cells to stimulate osteoclast activity, the main concept of our work is that the stimulatory action of these effectors was, at least in part, mediated through upregulation of PTP-oc expression via RANKL-independent mechanism.

In as much as our results support our model that resorption effectors regulate osteoclast activity in part through transcriptional regulation of PTP-oc expression, there is circumstantial evidence that some effectors could also have direct effects on the enzymatic activity of PTP-oc. Accordingly, while the suppressive effect of PTH, $1,25(OH)_2D_3$, and PGE_2 on PTP-oc expression was $\sim$ twofold, the effects of these effectors on c-Src PY527 level and corresponding PTK activity were much higher (four- to eightfold). If the dephosphorylation of c-Src PY527 in osteoclasts were indeed mediated by PTP-oc, these findings could be interpreted as that PTH, $1,25(OH)_2D_3$, PGE_2, and/or other effectors may also stimulate the enzymatic activity of PTP-oc. There is abundance of evidence that the enzymatic activity of a PTP can be regulated. Accordingly, $1,25(OH)_2D_3$ has been shown to stimulate the activity of a PTP in HL-60 cells [Uchida et al., 1993]. The enzymatic activity of PTP-oc is shown to be negatively regulated by Pim-1 kinase in vitro [Wang et al., 2001]. Also, bisphosphonates are potent inhibitors of PTPs in osteoclasts [Schmidt et al., 1996; Murakami et al., 1997; Opas et al., 1997].

The second aspect of our model is that PTP-oc dephosphorylates PY527 of c-Src, which then activates the c-Src PTK activity in osteoclasts. Consistent with this hypothesis, the recombinant GST–PTP-oc fusion protein was able to dephosphorylate PY-527-containing c-Src peptide in vitro in a time- and dose-dependent manner. In addition, PTH, $1,25(OH)_2D_3$, and PGE_2, at a concentration, which upregulated

PTP-oc expression, each also significantly decreased the PY527 level and increased the PTK activity of c-Src in rabbit osteoclasts. Conversely, calcitonin and alendronate, each of which reduced the PTP-oc level, also markedly increased the PY527 level of c-Src that was accompanied by a decrease in its PTK activity. Although we have not established the cause–effect relationship between the effector-induced changes in PTP-oc expression and the effector-mediated c-Src activation, these findings provide strong associative evidence for the second aspect of our model that PTP-oc dephosphorylates PY527 of c-Src, leading to the activation of its PTK activity.

There is no convenient approach to directly test the third aspect of our model that PY527 of c-Src is indeed a cellular substrate of PTP-oc. However, if PY527 c-Src were indeed a substrate of PTP-oc, c-Src in osteoclasts should forms a tight complex with PTP-oc that can be co-immunoprecipitated with PTP-oc. Moreover, the overall PY527 level of the immunoprecipitated c-Src should be reduced when the PTP-oc expression was increased by resorption activators. Conversely, it should be increased when the PTP-oc expression was reduced by resorption inhibitors. Accordingly, our findings that c-Src was co-immunoprecipitated with PTP-oc and that the amounts of c-Src immunoprecipitated with PTP-oc were increased by resorption inhibitors and reduced by resorption activators provide circumstantial evidence for this aspect of our model. Although c-Src is known to bind a number of cellular proteins through its SH2 (a specialized PY binding motif) and/or SH3 (a specialized binding motif for proline-rich protein) domain, the association between c-Src and PTP-oc is probably not through interactions between PTP-oc and the SH2 and/or SH3 domain of c-Src, since PTP-oc is not tyrosyl phosphorylated and does not contain proline-rich domains [Wu et al., 1996]. Thus, we tentatively conclude that the association between c-Src and PTP-oc is related to substrate binding. The fact that PTP-oc forms a tight and specific complex with c-Src in osteoclasts is consistent with our hypothesis that PTP-oc dephosphorylates PY-527 of c-Src in osteoclasts.

In addition to c-Src, a number of members of the c-Src signaling pathway [Migliaccio et al., 1998] were also co-immunoprecipated with PTP-oc. c-Src has been shown to bind Shc [Sato et al., 2002] and Grb2 [Kimber et al., 2000] through its SH2 domain. It is possible that the co-immunoprecipitation of at least some of other PY proteins with PTP-oc could be mediated through indirect interactions between these proteins and c-Src. A circumstantial evidence for our conclusion that c-Src, but not other co-immunoprecipitated PY proteins, is a potential cellular substrate of PTP-oc in osteoclasts is that each of the test resorption activator (PTH, $1,25(OH)_2D_3$, PGE_2) reduced the overall PY level of only the co-immunoprecipitated c-Src but not the other co-immunoprecipitated proteins (Erk1/2, Shc, Grb2, and rasGAP) and that the test resorption inhibitors, calcitonin and alendronate, increased the overall PY level of only the co-immunoprecipitated c-Src. The fact that the recombinant GST–PTP-oc fusion protein was able to dephosphorylate the PY527-containing c-Src peptide in vitro (data not shown) further supports the possibility that c-Src and not the other test signaling proteins, including Erk1/2, is a potential cellular substrate of PTP-oc in rabbit osteoclasts.

There is compelling evidence that the PTK activity of c-Src and its signaling pathway is required for the spreading and migration of osteoclasts, since osteoclasts derived from c-Src deficient mice attached but did not spread on bone surface and inhibition of the c-Src PTK activity prevented the spreading of osteoclasts [Insogna et al., 1997; Lakkakorpi et al., 2001, 2003]. The spreading and migration of osteoclasts on bone surfaces are essential for osteoclastic resorption and also determine the size of the resorption pits. Accordingly, it is possible that the PTP-oc-induced upregulation of osteoclastic resorption is mediated at least in part through the c-Src-dependent upregulation of spreading and migration of osteoclasts. Our findings that the PTP-oc antisense oligo treatment significantly reduced the average size of resorption pit are consistent with this speculation. However, the PTP-oc antisense oligo treatment also significantly reduced the average depth of the resorption pits. Together with our previous observations that osteoclast-like cells derived from U937 cell clones overexpressing wild-type PTP-oc also produced significantly deeper resorption pits and osteoclast-like cells derived from U937 cell clones overexpressing phosphatase-dead PTP-oc yielded significantly shallower resorption pits [Amoui et al., 2004], our results clearly demonstrated that the PTP-oc regulates not only the size but also the

depth of resorption pits. This would suggest that the c-Src-regulated osteoclastic resorption is mediated not only through enhanced osteoclast spreading and migration, but also through an increase in the bone resorption activity. In support of this speculation, is the fact that osteoclasts of c-Src knockout mice were not only unable to spread on bone surface but also unable to create resorption pits [Soriano et al., 1991]. Alternatively, PTP-oc may regulate bone resorption activity of osteoclasts through c-Src-independent mechanisms. Regardless of how PTP-oc acts to regulate osteoclastic resorption, the findings of this study provide additional support for our conclusion that PTP-oc is a positive regulator of the c-Src PTK activity and the bone resorption activity of osteoclasts.

Consistent with our premise that one or more transmembrane PTPs in osteoclasts may act as positive regulators of osteoclast functions, Chiusaroli et al. [2004] have recently demonstrated that mice deficient in the receptor type PTP-ε exhibited increased trabecular bone mass due to cell-specific defects in osteoclast activity and that osteoclasts derived from PTP-ε deficient bone-marrow precursors were poorly functional in resorbing bone in vitro. These observations indicate that, similar to PTP-oc, PTP-ε also functions as a positive regulator of osteoclast activity. However, there are two significant differences between the signaling pathway of PTP-oc and that of PTP-ε. First, our data suggest that PTP-oc regulates osteoclast activity at least in part through the c-Src signaling pathway. Although there is evidence that PTP-ε activates c-Src in Neu-induced mammary tumor cells [Gil-Henn and Elson, 2003], the c-Src signaling pathway does not appear to be involved in the PTP-ε-dependent regulation of osteoclast function, since loss of PTP-ε in osteoclasts did not cause significant disruption of the c-Src signaling pathway in osteoclasts [Chiusaroli et al., 2004]. Second, there is evidence that PTP-ε plays an important role in inhibiting the Erk1/2 signaling pathway in fibroblasts and mammary tumor cells, since loss of PTP-ε expression in cells derived from PTP-ε knockout mice showed increased Erk1/2 activation and phosphorylation [Toledano-Katchalski et al., 2003]. In contrast, this study indicates that PTP-oc does not appear to have a regulatory role in Erk1/2 phosphorylation or activation in rabbit osteoclasts. Consequently, although both PTP-oc and PTP-ε act as positive regulators of osteoclast function, these two transmembrane PTPs may act through different mechanisms to upregulate osteoclast activity and functions.

In conclusion, we have obtained three lines of evidence supporting our model (Fig. 10) that PTP-oc acts to dephosphorylate PY527 of c-Src in osteoclasts, which leads to activation of the PTK activity of c-Src and its associated signal transduction pathway, which in turn results in the activation of osteoclasts and increased osteoclastic resorption. These data together indicate that PTP-oc is a potential positive regulator of the c-Src PTK activity and, as such, is a positive regulator of osteoclast activity.

ACKNOWLEDGMENTS

We thank Christina Goodwin for her technical assistance. We acknowledge the assistance of Dr. Xiao Mao and Lora Green of Loma Linda University for their assistance in the confocal measurements. All work was performed in facilities provided by the Department of Veterans Affairs. This work was supported in part by a research grant from the National Institutes of Dental and Craniofacial Research (RO1 DE13097 to K.-H.W.L.) and a VA Merit Review grant (to K.-H.W.L).

REFERENCES

Aguiar RCT, Yakushijin Y, Kharbanda S, Tiwari S, Freeman GJ, Shipp MA. 1999. PTPROt: An alternatively spliced and developmentally regulated B-lymphoid phosphatase that promotes G0/G1 arrest. Blood 94:2403–2413.

Amoui M, Baylink DJ, Tillman JB, Lau K-HW. 2003. Expression of a structurally unique osteoclastic protein-tyrosine phosphatase is driven by an alternative intronic, cell-type-specific promoter. J Biol Chem 278:44273–44280.

Amoui M, Suhr SM, Baylink DJ, Lau K-HW. 2004. An osteoclastic protein-tyrosine phosphatase may play a role in the differentiation and activity of human monocytic U-937 cell-derived, osteoclast-like cells. Am J Physiol Cell Physiol 287:C874–C884.

Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, Carter-Su C. 1993. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74:237–244.

Blair HC, Jordan SE, Peterson TG, Barnes S. 1996. Variable effects of tyrosine kinase inhibitors on avian osteoclastic activity and reduction of bone loss in ovariectomized rats. J Cell Biochem 61:629–637.

Boyce BF, Yoneda T, Lowe C, Soriano P, Mundy GR. 1992. Requirement of $pp60^{c\text{-}src}$ expression for osteoclasts to form ruffled borders and resorb bone in mice. J Clin Invest 90:1622–1627.

Brickell PM. 1992. The p60$^{c\text{-}src}$ family of protein-tyrosine kinases: Structure, regulation and function. Crit Rev Oncogenesis 3:401–446.

Chappel J, Ross FP, Abu-Amer Y, Shaw A, Teitelbaum SL. 1997. 1,25-dihydroxyvitamin D_3 regulates pp60$^{c\text{-}src}$ activity and expression of a pp60$^{c\text{-}src}$ activating phosphatase. J Cell Biochem 67:432–438.

Chiusaroli R, Knobler H, Luxenburg C, Sanjay A, Granot-Attas S, Tiran Z, Miyazaki T, Harmelin A, Baron R, Elson A. 2004. Tyrosine phosphatase epsilon is a positive regulator of osteoclast function in vitro and in vivo. Mol Biol Cell 15:234–244.

Faucheux C, Horton MA, Price JS. 2002. Nuclear localization of type I parathyroid hormone/parathyroid hormone-related protein receptors in deer antler osteoclasts: Evidence for parathyroid hormone-related protein and receptor activator of NF-κB-dependent effects on osteoclast formation in regenerating mammalian bone. J Bone Miner Res 17:455–464.

Gay CV, Zheng B, Gilman VR. 2003. Co-detection of PTH/PTHrP receptor and tartrate resistant acid phosphatase in osteoclasts. J Cell Biochem 89:902–908.

Gil-Henn H, Elson A. 2003. Tyrosine phosphatase-ε activates Src and supports the transformed phenotype of Neu-induced mammary tumor cells. J Biol Chem 278:15579–15586.

Hall TJ, Schaeublin M, Missbach M. 1994. Evidence that c-src is involved in the process of osteoclastic bone resorption. Biochem Biophys Res Commun 199:1237–1244.

Harder KW, Owen P, Wong LKH, Aebersold R, Clark-Lewis I, Jirik FR. 1994. Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase β (HPTPβ) using synthetic phosphopeptides. Biochem J 298:395–401.

Insogna K, Tanaka S, Neff L, Horne W, Levy J, Baron R. 1997. Role of c-Src in cellular events associated with colony-stimulating factor-1-induced spreading in osteoclasts. Mol Reprod Dev 46:104–108.

Kimber MS, Nachman J, Cunningham AM, Gish GD, Pawson T, Pai EF. 2000. Structural basis for specificity switching of the Src SH2 domain. Mol Cell 5:1043–1049.

Lakkakorpi PT, Nakamura I, Young M, Lipfert L, Rodan GA, Duong LT. 2001. Abnormal localization and hyperclustering of αvβ3 integrins and associated proteins in Src-deficient or tyrphostin A9-treated osteoclasts. J Cell Sci 114(Pt 1):149–160.

Lakkakorpi PT, Bett AJ, Lipfert L, Rodan GA, Duong LT. 2003. PYK2 autophosphorylation, but not kinase activity, is necessary for adhesion-induced association with c-Src, osteoclast spreading, and bone resorption. J Biol Chem 278:11502–11512.

Langub MC, Reinhardt TA, Horst RL, Malluche HH, Koszewski NJ. 2000. Characterization of vitamin D receptor immunoreactivity in human bone cells. Bone 27: 383–387.

Langub MC, Monier-Faugere MC, Qi Q, Geng Z, Koszewski NJ, Malluche HH. 2001. Parathyroid hormone/parathyroid hormone-related peptide type 1 receptor in human bone. J Bone Miner Res 16:448–456.

Migliaccio A, Piccolo D, Castoria G, Di Domenico M, Bilancio A, Lombardi M, Gong W, Beato M, Auricchio F. 1998. Activation of the Src/p21ras/Erk pathway by progesterone receptor via cross-talk with estrogen receptor. EMBO J 17:2008–2018.

Missabach M, Jeschke M, Feyen J, Muller K, Glatt M, Green J, Susa M. 1999. A novel inhibitor of the tyrosine kinase Src suppresses phosphorylation of its major cellular substrates and reduces bone resorption in vitro and in rodent models in vivo. Bone 24:437–439.

Murakami H, Takahashi N, Tanaka S, Nakamura I, Udagawa N, Nakajo S, Nakaya K, Abe M, Yuda Y, Konno F, Barbier A, Suda T. 1997. Tiludronate inhibits protein tyrosine phosphatase activity in osteoclasts. Bone 20:399–404.

Nakashima M, Nakayama T, Ohtsuru A, Fukada E, Niino D, Yamazumi K, Naito S, Ito M, Sekine I. 2003. Expression of parathyroid hormone (PTH)-related peptide (PTHrP) and PTH/PTHrP receptor in osteoclast-like giant cells. Pathol Res Pract 199:85–92.

Opas EE, Rutledge SJ, Golub E, Stern A, Zimolo Z, Rodan GA, Schmidt A. 1997. Alendronate inhibition of protein-tyrosine-phosphatase-meg 1. Biochem Pharm 54:721–727.

Palmqvist P, Persson E, Conaway HH, Lerner UH. 2002. IL-6, leukemia inhibitory factor, and oncostatin M stimulate bone resorption and regulate the expression of receptor activator of NF-κB ligand, osteoprotegerin, and receptor activator of NF-κB in mouse calvariae. J Immunol 169:3353–3362.

Sarrazin P, Hackett JA, Fortier I, Gallant MA, de Brum-Fernandes A. 2004. Role of EP3 and EP4 prostaglandin receptors in reorganization of the cytoskeleton in mature human osteoclasts. J Rheumatol 31:1598–1606.

Sato K, Nagao T, Kakumoto M, Kimoto M, Otsuki T, Iwasaki T, Tokmakov AA, Owada K, Fukami Y. 2002. Adaptor protein Shc is an isoform-specific direct activator of the tyrosine kinase c-Src. J Biol Chem 277:29568–29576.

Schmidt A, Rutledge SJ, Endo N, Opas EE, Tanaka H, Wesolowski G, Leu CT, Huang Z, Ramachandaran C, Rodan SB, Rodan GA. 1996. Protein-tyrosine phosphatase activity regulates osteoclast formation and function: Inhibition by alendronate. Proc Natl Acad Sci USA 93: 3068–3073.

Schwartzberg PL, Xing L, Hoffmann O, Lowell CA, Garrett L, Boyce BF, Varmus HE. 1997. Rescue of osteoclast function by transgenic expression of kinase-deficient Src in src$^{-/-}$ mutant mice. Genes Dev 11:2835–2844.

Seimiya H, Sawabe T, Inazawa J, Tsuruo T. 1995. Cloning, expression and chromosomal localization of a novel gene for protein tyrosine phosphatase (PTP-U2) induced by various differentiation-inducing agents. Oncogene 10: 1731–1738.

Shiotani A, Takami M, Itoh K, Shibasaki Y, Sasaki T. 2002. Regulation of osteoclast differentiation and function by receptor activator of NFκB ligand and osteoprotegerin. Anat Rec 268:137–146.

Soriano P, Montgomery C, Geske R, Bradley A. 1991. Targeted disruption of the c-src proto-oncogene leads to osteopetrosis in mice. Cell 64:693–702.

Suda T, Nakamura I, Jimi E, Takahashi N. 1997. Regulation of osteoclast function. J Bone Miner Res 12:869–879.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. 1999. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. Endocr Rev 20:345–357.

Suhr SM, Pamula S, Baylink DJ, Lau K-HW. 2001. Antisense oligodeoxynucleotide evidence that a unique osteoclastic protein-tyrosine phosphatase is essential for osteoclastic resorption. J Bone Miner Res 16:1795–1803.

Tanake S, Takahashi N, Udagawa N, Sasaki T, Fukui Y, Kurokawa T, Suda T. 1992. Osteoclasts express high levels of $p60^{c\text{-}src}$, preferentially on ruffled border membranes. FEBS Lett 323:85–89.

Tezuka K, Sato T, Kamioka H, Nijweide PJ, Tanaka K, Matsou T, Ohta M, Kurihara N, Hakeda Y, Kumegawa M. 1992. Identification of osteopontin in isolated rabbit osteoclasts. Biochem Biophys Res Commun 186:911–917.

Toledano-Katchalski H, Kraut J, Sines T, Granot-Attas S, Shohat G, Gil-Henn H, Yung Y, Elson A. 2003. Protein tyrosine phosphatase ε inhibits signaling by mitogen-activated protein kinases. Mol Cancer Res 1:541–550.

Uchida T, Matozaki T, Matsuda K, Suzuki T, Matozaki S, Nakano O, Wada K, Konda Y, Sakamoto C, Kasuga M. 1993. Phorbol ester stimulates the activity of a protein tyrosine phosphatase containing SH2 domains (PTP1C) in HL-60 leukemia cells by increasing gene expression. J Biol Chem 268:11845–11850.

Wang Z, Bhattacharya N, Meyer MK, Seimiya H, Tsuruo T, Tonani JA, Magnuson NS. 2001. Pim-1 negatively regulates the activity of PTP-U2S phosphatase and influences terminal differentiation and apoptosis of monoblastoid leukemia cells. Arch Biochem Biophys 390:9–18.

Wharram BL, Goyal M, Gillespie PJ, Wiggins JE, Kershaw DB, Holzman LB, Dysko RC, Saunders TL, Samuelson LC, Wiggins RC. 2000. Altered podocyte structure in GLEPP1 (Ptpro)-deficient mice associated with hypertension and low glomerular filtration rate. J Clin Invest 106:1281–1290.

Wiggins RC, Wiggins JE, Goyal M, Wharram BL, Thomas PE. 1995. Molecular cloning of cDNA encoding human GLEPP1, a membrane protein tyrosine phosphatase: Characterization of the GLEPP1 protein distribution in human kidney and assignment of the GLEPP1 gene to human chromosome 12p12-p13. Genomics 27:174–181.

Wu L-W, Baylink DJ, Lau K-HW. 1996. Molecular cloning and expression of a unique rabbit osteoclastic phosphotyrosyl phosphatase. Biochem J 316:515–523.

Yoneda T, Lowe C, Lee C-H, Gutierrez G, Niewolna M, Williams PJ, Izbicka E, Uehara Y, Mundy GR. 1993. Herbimycin A, a $pp60^{c\text{-}src}$ tyrosine kinase inhibitor, inhibits osteoclast bone resorption in vitro and hypercalcemia in vivo. J Clin Invest 91:2791–2795.

Yoneda T, Niewolna M, Lowe C, Izbicka E, Mundy GR. 1994. Hormonal regulation of $pp60^{c\text{-}src}$ expression during osteoclast formation in vitro. Mol Endocrinol 7:1237–1244.