Molecular cloning and expression of a unique rabbit osteoclastic phosphotyrosyl phosphatase

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Tyrosyl phosphorylation plays an important regulatory role in osteoclast formation and activity. Phosphotyrosyl phosphatases (PTPs), in addition to tyrosyl kinases, are key determinants of intracellular tyrosyl phosphorylation levels. To identify the PTP that might play an important regulatory role in osteoclasts, we sought to clone an osteoclast-specific PTP. A putative full-length clone encoding a unique PTP (referred to as PTP-oc) was isolated from a 10-day-old rabbit osteoclastic cDNA library and sequenced. A single open reading frame predicts a protein with 405 amino acid residues containing a putative extracellular domain, a single transmembrane region, and an intracellular portion. PTP-oc is structurally unique in that, unlike most known transmembrane PTPs, it has a short extracellular region (eight residues), lacks a signal peptide proximal to the N-terminus, and contains only a single 'PTP catalytic domain'. The PTP catalytic domain shows 45–50% sequence identity with the catalytic domain of human HPTPβ and with the first catalytic domain of LCA. The PTP-oc gene exists as a single copy in the rabbit genome. The corresponding mRNA (3.8 kb) is expressed in osteoclasts but not in other bone-derived cells (e.g. osteoblasts and stromal cells). The 3.8 kb PTP-oc mRNA transcript was also expressed in the rabbit brain, kidney, and spleen. However, the brain and kidney, but not osteoclasts or spleen, also expressed a larger transcript (6.5 kb). The PTP catadymic domain of PTP-oc was expressed as a GST-cPTP-oc fusion protein. In vitro phosphatase assays indicated that the purified fusion protein exhibited phosphatase activities at neutral pH values toward p-nitrophenyl phosphate, phosphotyrosyl Raytide, and phosphotyrosyl histone, whereas it had no appreciable activity toward phosphoserine casein. In summary, we have: (a) cloned and sequenced the putative full-length cDNA of a unique PTP (PTP-oc) from rabbit osteoclasts; (b) shown that the mature 3.8 kb PTP-oc mRNA was expressed primarily in osteoclasts and the spleen; and (c) shown that the PTP-oc fusion protein exhibited a phosphotyrosine-specific phosphatase activity. In conclusion, PTP-oc represents a structurally unique subfamily of transmembrane PTPs.

INTRODUCTION

Bone resorption is required for bone growth (modelling), remodelling and repair processes. The bone resorption process is mediated by osteoclasts [1]. Recent observations indicated that at least two members of the protein tyrosyl kinase (PTK) family may be involved in normal regulation of osteoclast formation and activity. Studies with the op/op variant of murine osteopetrosis have shown that production of macrophage-colony stimulating factor (M-CSF) (colony stimulating factor-1) and activation of its receptor are required for normal osteoclast formation [2–4]. The M-CSF receptor, which is encoded by the proto-oncogene c-fms, contains an intrinsic PTK activity [5]. In addition, recent studies with the c-src gene knock-out mice have indicated that the c-src PTK activity is essential for the manifestation of osteoclastic bone resorption and ruffled border formation, but not for osteoclast formation [6,7]. The ruffled border is the highly specialized area of the osteoclast membrane, where bone resorption occurs. The pp60^{c-src} PTK activity, which is regulated by its tyrosyl phosphorylation status, has been shown to correlate with the number of bone-resorbing osteoclasts [8]. Accordingly, these findings strongly suggest that the intracellular protein tyrosyl phosphorylation status in osteoclasts could play an important regulatory role with respect to osteoclast formation and activity.

The steady-state cellular level of protein tyrosyl phosphorylation is controlled by the balance of the PTK and phosphotyrosyl phosphatase (PTP) activities. At one time, tyrosyl phosphorylation was thought to be regulated largely by PTK activities. However, a large body of evidence has accumulated to indicate that PTPs do not play a mere counteracting role to the action of PTKs, but instead are important determinants of various cellular functions [9–12]. Like PTKs, PTPs are widely distributed in various mammalian cells and tissues, and belong to a superfamily of distinct but structurally related enzymes [13,14]. There are at least two distinct PTP families: one family consists of cytosolic enzymes with a single catalytic domain (e.g. human PTP-1B, T-cell PTP, PTP-2C, etc.) [15–17]; and the other family are transmembrane PTPs which structurally are composed of extracellular, transmembrane and cytoplasmic domains. The cytoplasmic portion of the known transmembrane PTPs contains two tandem catalytic domains, with the exception of HPTPβ and DPTP10D, both of which contain only a single catalytic domain [18–20]. Recent studies suggest that the membrane-proximal catalytic domain (PDI) is catalytically active, whereas the membrane-distal catalytic domain (PDII) by itself has no measurable enzymic activity but may have regulatory functions [21,22]. While the cytoplasmic core phosphatase domains of PTPs are highly conserved, the extracellular domains of the receptor-like PTPs are unrelated to each other [23,24]. Although

Abbreviations used: DTT, dithiothreitol; FN-III, fibronectin type-III; GAPDH, glyceraldehyde-3'-phosphate dehydrogenase; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactopyranoside; M-CSF, macrophage-colony stimulating factor; ORF, open reading frame; pNPP, p-nitrophenyl phosphate; PTK, protein tyrosyl kinase; PTP, phosphotyrosyl phosphatase; RT-PCR, reverse-transcription PCR; UTR, untranslated region.

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The complete cDNA sequence has been deposited in GeneBank, and received the accession number U32587.
numerous PTPs have been cloned from various cell types, the identity of the PTPs in osteoclasts have not been investigated.

While the importance of PTKs, such as pp60c-src and c-fms, in osteoclasts has been well documented [2–8], the potential involvement of PTPs in osteoclast formation and activity has been less clear. Accordingly, as an initial approach to examining the identity and potential functions of PTPs in osteoclasts, we sought to identify and clone a unique PTP in osteoclasts. We reasoned that an osteoclast-specific PTP could have specialized functions in osteoclasts. In this paper, we report the cloning and complete sequence of the full-length cDNA of a novel rabbit osteoclastic PTP, termed PTP-oc.

EXPERIMENTAL

Materials

All oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, U.S.A.). Superscript II reverse transcriptase and dithiothreitol (DTT) were from Gibco/BRL Life Technologies (Grand Island, NY, U.S.A.). Restriction enzymes, large fragment of DNA polymerase I (Klenow fragment) and RNAsin were purchased from Promega (Los Angeles, CA, U.S.A.). QIAIEN plasmid kits were obtained from QIAIEN, Inc. (Chatsworth, CA, U.S.A.). SDS was ordered from National Diagnostics, Inc. (Atlanta, GA, U.S.A.). Sequence kits and formamide were from United States Biochemical (Cleveland, OH, U.S.A.). Vent (exo-) DNA polymerase and EcoRI linker were products of New England Biolabs (Beverly, MA, U.S.A.). Nitrocellulose and MagnaGraph nylon filters were from Micro Separations Inc. (Westborough, MA, U.S.A.). PMSF, BSA (fraction V), isopropl β-D-thiogalactopyranoside (IPTG), p-nitrophenyl phosphate (pNPP), glutathione (reduced form), antipain, leupeptin, pepstatin, histone type II A, casern, the catalytic subunit of protein kinase A, and Norit A were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was from the American Type Culture Collection (Rockville, MD, U.S.A.). Raytide and pp60c-src were obtained from Oncogene Science, Inc. (Uniondale, NY, U.S.A.). [γ-32P]ATP (7000 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were purchased from DuPont NEN (Boston, MA, U.S.A.). dNTP, pGEX-5X-3 vector and glutathione-Sepharose 4B were from Pharmacia Biotech Inc. (Piscataway, NJ, U.S.A.). Bradford protein reagent was from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other reagents were of either reagent or molecular biology grade and were obtained from Fisher Scientific (Tustin, CA, U.S.A.) or Sigma Chemical Co.

Cell cultures

Rabbit osteoclasts were isolated from bones of 10-day-old rabbits as described previously [25]. Osteoblasts were obtained by outgrowth of cells from explants of bone chips of 10-day-old rabbits. Stromal cells were prepared from bone marrows of 10-day-old rabbits.

Reverse-transcription PCR (RT-PCR)

For RT-PCR, degenerate oligonucleotide primers (oligos I and II) were designed from two conserved regions within the catalytic domains of the previously characterized PTPs. The sense oligo I (5'-AARUGYSMCARAYUGGCC) corresponded to the amino acid sequence KC(A/D/H)QYW, whereas the antisense oligo II (5'-CCNYAYRCBCGCTRACGAT) corresponded to (I/V)HCSAG(V/A)GGSA. Total RNA from the isolated rabbit osteoclasts was used as the template for the reverse transcription. Reverse transcription was initiated by adding 200 units of Superscript II reverse transcriptase to a reaction mixture of 20 µl containing 50 µM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 0.5 µg of total RNA, 4 µM oligo II, 20 units of RNAsin, 10 mM DTT and 1 mM dNTP. The reaction was carried out at 42 °C for 20 min. The first-strand cDNA was purified by phenol–chloroform extraction, and recovered with ethanol–ammonium acetate precipitation.

The PCR was carried out in a 50 µl reaction mixture containing 10 mM KCl, 20 mM Tris/HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1 % Triton X-100, 2 units of Vent (exo-) DNA polymerase, 0.8 mM dNTP, 0.1 mg/ml BSA, 4 µM each of oligos I and II and with the first-strand cDNA derived from rabbit osteoclast mRNA as the template. Before cycling, the reaction was subjected to a hot start for 5 min at 100 °C, then 2 min at 49 °C, before the addition of Vent (Exo-) polymerase. This procedure was followed by 30 cycles of extension for 1 min at 74 °C, denaturing for 1 min at 94 °C, and annealing for 1 min at 49 °C. The resulting PCR products were purified by phenol–chloroform extraction, blunted by Klenow then subcloned into the Smal-digested pUC119 vector.

Nucleotide sequence determination

Plasmids were prepared by either the modified alkaline lysis method [26] or QAIEN plasmid isolation kits. DNA sequences were determined by the dideoxy nucleotide chain-termination method [27] with a series of specific synthetic oligonucleotides as primers (17–20-mers).

Analyses of nucleotide and amino acid sequences

All DNA and deduced protein sequence searches were done with the release 6.85 of PC/GENE sequence analysis software package developed by IntelliGenetics (Mountain View, CA, U.S.A.). The EMBL 41 and SWISS-PROT 41 databases were searched, respectively, with the Lipman–Pearson FASTA program [28]. Hydrophathy index computation for PTP-oc was based on the method developed by Klein et al. [29]. The alignment of multiple protein sequences were achieved with the CLUSTAL program using the Dayhoff’s matrix for scoring and a K-tuple value of 2 and a gap penalty of 10 [30].

Screening of the rabbit osteoclastic cDNA library and isolation of cDNA clones

One of the PCR subclones (i.e. PCR1), which encoded a novel PTP fragment, was used as the probe to screen a λZAPII rabbit osteoclastic cDNA library (a generous gift from Dr. M. Kumegawa; [25]). Nitrocellulose filter lifts of the phage plates, each with 20000 plaques/100 mm², were washed with 5 × SSC, 0.5 % SDS and 1 mM EDTA (pH 8) at 42 °C for 2 h prior to hybridization (SSC: 0.15 M NaCl, 0.015 M sodium citrate). The nitrocellulose membranes were hybridized overnight at 42 °C in a hybridization buffer consisting of 5 × SSPE, 5 × Denhardt’s solution, 0.1 % SDS, 100 µg/ml salmon sperm DNA and 10⁶ c.p.m./ml of probe [SSPE: 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA]. The filters were then washed in 2 × SSC/0.1 % SDS at room temperature four times, each for 10 min, followed by washing once in 1 × SSC/0.1 % SDS at 65 °C for 80 min. The final washes were with 0.2 × SSC/0.1 % SDS at 65 °C twice, each for 90 min. Putative positive phase clones were plaque-purified. The pBluescript-derived plasmids containing the osteoclastic cDNA inserts were recovered from λZAPII phage by in vitro excision according to the manufacturer’s
Production and purification of PTP-oc fusion proteins

For production of bacterial glutathione S-transferase (GST) fusion proteins containing the catalytic region of PTP-oc (i.e. cPTP-oc), the DNA fragment encoding the putative catalytic domain (nucleotides 665–1538) was cloned in-frame to the gene encoding the catalytic domain in the PTP-oc cDNA clone. The 0.9 kb DNA fragment containing the putative catalytic domain was generated by ligation of the EcoRI site introduced at the 5′ end of the cDNA insert (5′ cDNA probe in Figure 3). The same filter was stripped and rehybridized to the labelled 1.7 kb fragment derived from the 3′-end of the cDNA insert (3′ cDNA probe in Figure 3). Both cDNA fragments were labelled by the random-priming method [32]. Hybridization of DNA blots were carried out overnight at 42 °C in a buffer containing 50 % formamide, 7 % SDS, 0.25 M sodium phosphate (pH 7.2), 0.25 M NaCl, 1 mM EDTA (pH 8), 200 μg/ml denatured salmon sperm DNA and [32P]-labelled probe (106–107 c.p.m./ml). The filters were washed with 2 × SSPE/0.1 % SDS at room temperature, and twice at 50 °C, each time for 20 min. The final washes were carried out at 50 °C in 0.2 × SSPE/0.1 % SDS twice, each for 30 min. Autoradiography was performed with a DuPont Cronex III intensifying screen at −80 °C.

Phosphatase assays

The phosphatase activity of the GST–cPTP-oc fusion protein was assayed with (a) pNPP, (b) [γ32P]tyrosyl-phosphorylated Raytide, and (c) [3H]tyrosyl-phosphorylated histone as the in vitro substrate at neutral pH as previously described [20,34–36]. To determine the specificity of the phosphatase activity, [3H]serine-phosphorylated casein was included as a substrate.

(a) pNPP phosphatase assay

The reaction mixture consisted of 20 mM Tris/HCl (pH 7.2), 0.1 % 2-mercaptoethanol, 0.05 % Triton X-100, 0.1 mg/ml BSA, an aliquot of fusion protein and pNPP at the indicated concentration, in a final volume of 100 μl. The reaction was carried out at 30 °C for 0–20 min and terminated by addition of 1 ml of 0.2 M NaOH. Absorbance at 410 nm was determined and a molar absorption coefficient of 1.78 × 105 M−1 cm−1 was used to calculate the concentration of the p-nitrophenolate ion produced in the reaction.

(b) Raytide phosphatase assay

Raytide was phosphorylated at the tyrosine residue with [γ32P]ATP and pp60src PTK as described by the supplier. Following the phosphorylation, the [32P]-labelled Raytide was purified to remove the unincorporated [γ32P]ATP as described elsewhere [37]. The dephosphorylation reaction of [32P]tyrosine-phosphorylated Raytide was initiated by adding an aliquot of purified fusion protein in a final volume of 50 μl of reaction mixture including 25 mM Hepes (pH 7.6), 5 mM EDTA (pH 8.0), 10 mM DTT and 32P-labelled Raytide [(1–2) × 108 c.p.m.]. The reaction was carried out at 30 °C for the indicated length of time, and was terminated by addition of 0.75 ml of a charcoal mixture [0.9 M HCl/90 mM sodium pyrophosphate/2 mM NaH2PO4 and 1.4 % (w/v) Norit A]. Following centrifugation, 0.1 ml of supernatant was spotted on a Whatman glass filter circle and [32P], was measured by Čerenkov counting. The relative enzyme activity was reported as the percentage of [32P]P, released.

(c) Phosphoprotein phosphatase assay

Phosphotyrosyl protein phosphatase assay was measured with tyrosyl phosphorylated histone. Histone type IIA was phosphorylated with [γ32P]ATP by pp60src PTK as previously described [37]. The phosphoseryl protein phosphatase activity was assayed with 32P-labelled casein, which was phosphorylated at the serine/threonine residues by the catalytic subunit of protein kinase A as described in [37]. Dephosphorylation of phosphorylated proteins was performed under the same conditions as that for Raytide with the exception that 30000 c.p.m. of 32P-labelled histone or 32P-labelled casein, instead of Raytide,
was used as the substrate. The protein phosphatase assay was terminated by adding 100 µl of ice-cold 20 % trichloroacetic acid and 100 µg of BSA into the reaction mixture. Following centrifugation, 50 µl of the supernatant was spotted on a Whatman glass filter circle and [32P]Pi was measured by Cerenkov counting. The relative enzyme activity was reported as the percentage of [32P]Pi released.

Other methods
Protein concentrations were determined by the method of Bradford [38] using BSA as the standard. SDS-PAGE was performed as described elsewhere [31].

RESULTS

Molecular cloning of PTP-oc cDNA
Degenerate oligonucleotides which corresponded to the two conserved motifs [i.e. KC(D/H)QYP and (I/V)CSAG(V/A)] within the ‘PTP catalytic domain’ were synthesized and used in a RT-PCR with rabbit osteoclast mRNA as the template. The PCR product was approx. 300 bp in length, which agreed with the anticipated nucleotide size between the two conserved motifs. The resulting PCR product was subcloned into pUC119 vector and sequenced. Of the 37 PCR subclones sequenced two (i.e. PCR1 and PCR12) were found to encode potentially novel members of the PTP family. To test whether the mRNA recognized by these putative novel PTP probes would display tissue-specific expression, Northern blot analysis of total RNA isolated from various rabbit tissues was performed. The cDNA fragments from PCR1 and PCR12 were each used as the probe for Northern hybridization. Both PCR1 and PCR12 probes hybridized with similar mRNA species, and showed identical tissue-specific expression patterns. In this regard, the probes hybridized with either a single or two mRNA transcripts in the spleen, brain and kidney, but not in thymus, heart, lung, liver, and haematopoietic blood cells (results not shown). Thus, the two PCR probes could be related and recognized the same PTP mRNA species. Accordingly, only the insert of PCR1 subclone was used as the probe to screen the rabbit osteoclast cDNA library.

One half of a million plaques were screened, and five positive cDNA clones were selected for further analysis. Restriction enzyme mapping and partial sequence analysis indicated that these clones could be related (results not shown). Because we are interested in cloning the full-length cDNA of the putative novel PTP, we isolated and purified the cDNA insert of clone 4-21, which contained the longest cDNA insert (i.e. approx. 4 kb), for sequence determination. This cDNA is referred to as PTP-oc.

Sequence analysis of PTP-oc
The complete nucleotide sequence of the PTP-oc cDNA was determined and confirmed by sequencing both strands. The nucleotide and the corresponding predicted amino acid sequences are shown in Figure 1. A single open reading frame (ORF) of 1215 bp was identified in this cDNA insert of 3623 bp in size. As observed in other mammalian PTPs [20,39,40], the PTP-oc cDNA sequence had two in-frame methionine codons (ATG), separated by nine nucleotides at the beginning of the ORF. The nucleotide sequence around the first ATG conforms to the canonical translation initiation signal, (A/G)CCATG [41]. The putative initiation methionine codon is preceded by several in-frame termination codons. The ORF was preceded by a 5' untranslated region (UTR) of 391 bp in length and followed by a relatively long 3' untranslated region (UTR) of 831 bp. The complete nucleotide sequence and predicted amino acid sequence of the PTP-oc are shown in Figure 1.
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Figure 2 Amino acid sequence alignment of PTP-oc with other mammalian PTPs

The catalytic domain (PD) sequences of the following PTPs are aligned: rabbit PTP-oc (146–394 amino acids), human HPTPβ (1722–1997 amino acids), human LAR (1359–1606 amino acids), human CD45 (669–919 amino acids), human PTP-1B (35–275 amino acids) and human PTP-2C (267–517 amino acids). For those PTPs with two catalytic domains, only the first catalytic domain (PDI) is used. Gaps (hyphens) are added for optimal alignment. The amino acid residues that are identical in at least five of six PTPs are in boldface font. Residues shared by all six sequences are indicated by asterisks below the sequences.

Figure 3 Restriction map of the rabbit PTP-oc cDNA

The restriction map of the rabbit cDNA which resides on the 3623 bp fragment is shown. The first nucleotide reported corresponds to the 5' end of the PTP-oc cDNA insert. The ORF of the PTP-oc protein is denoted with a black box, while the 5' and 3' UTRs are shown by thin lines. The thick bars indicate the length and position of 5' and 3' probes used in Northern and Southern blot hybridizations.

Sequence comparison between PTP-oc and other PTPs

Comparison and alignment of the predicted amino acid sequence of PTP-oc with the existing protein sequences in the up-to-date SWISS-PROT databank using the FASTA program indicated that the PTP-oc was most related to human transmembrane HPTPβ and Drosophila receptor-linked PTP10D. The sequence similarity (less than 50% identity) was found primarily in the regions containing the 'PTP catalytic domain'. Unlike most known transmembrane PTPs, which have two tandem catalytic domains in their cytoplasmic portions, the cytoplasmic domain of PTP-oc contains only a single 'PTP catalytic domain'. Thus far, the human transmembrane HPTPβ and Drosophila PTP10D are the only other known examples of a transmembrane PTP containing only a single catalytic domain in the cytoplasmic regions. Because the catalytic domains of cytosolic and transmembrane PTPs are known to be highly conserved, we compared the core phosphatase domains of the rabbit PTP-oc (shown in Figure 2) with those of three human receptor-like PTPs (i.e. HPTPβ, LAR:PDI, LCA:CD45:PDI) and two human cytosolic PTPs (i.e. PTP-1B, PTP-2C) [16,23,39,43,44]. The predicted catalytic domain of PTP-oc shares higher sequence identity with that of transmembrane PTPs (e.g. 48% with HPTPβ and 45% with LCA:CD45) than that of cytosolic PTPs (e.g. 38% with PTP-1B). While most transmembrane PTPs (except HPTPβ and HPTP2) have a large extracellular domain of several hundred residues which contains numerous potential glycosylation sites (i.e. N-X-S or N-X-T), Ig-like motifs, or fibronectin type-III (FN-III) motifs [23,36,44], the extracellular domain of PTP-oc is very short (i.e. eight residues), which shares no significant sequence similarity with the known transmembrane PTPs.

Tissue specificity of PTP-oc mRNA

To examine whether expression of PTP-oc mRNA is specific to osteoclasts but not other bone-derived cells, a 1.6 kb 5' cDNA probe (as shown in Figure 3) was used as a probe in the Northern blot analysis of total RNA isolated from osteoclasts, osteoblasts and the active site (i.e. HCSAGVGTG), which is highly conserved among the mammalian PTPs [13], was found within the putative ‘PTP catalytic domain’ of PTP-oc. These findings, taken together, are consistent with the interpretation that PTP-oc is a transmembrane PTP.

Molecular structure of PTP-oc

The ORF predicted a protein of 405 amino acid residues with a calculated molecular mass of 47276 Da. The hydropathy index computation analysis identified a single stretch of strongly hydrophobic amino acid residues (indicated in Figure 1 by underlining), a characteristic of a transmembrane domain. These 25 hydrophobic residues are followed by several basic residues (i.e. RKK), that are consistent with the stop-transfer signal associated with the membrane-spanning domains [42]. In contrast to the previously described generic transmembrane PTPs, the PTP-oc lacks at the N-terminus a signal peptide that targets the protein to the secretory pathway. The putative extracellular domain is very short (i.e. eight residues) and the cytoplasmic region is comprised of 372 residues. Several consensus motifs, including the two regions that were used to design degenerate oligonucleotides for RT-PCR, were identified in the cytoplasmic region. No SH2 domain was found. The ‘signature sequence’ of long (i.e. 1917 bp) 3'-UTR with stop codons in all three ORFs and a putative polyadenylation signal (AATAAA).
and bone-marrow stromal cells of 10-day-old rabbits. Figure 4 shows that a major transcript of approx. 3.8 kb was found in osteoclasts, but not in osteoblasts or bone-marrow stromal cells, indicating that the expression of the PTP-oc mRNA is specific for osteoclasts and not for other bone-derived cells. We should point out that much less osteoclast RNA (lane 1 in the bottom panel of Figure 4B) than RNAs from osteoclasts, osteoblasts and bone-marrow stromal cells were subjected to Northern blotting. The blot was hybridized with the 5'–PTP-oc cDNA probe (top panel), then with GAPDH cDNA probe (middle panel). The ethidium bromide-stained 18 S and 28 S rRNAs are indicated (bottom panel).

Figure 5 Southern analysis of rabbit osteoblast genomic DNA
Genomic DNA (10 µg) was digested with the indicated restriction enzymes. Digested DNA was transferred to a nylon filter and probed with a 32P-labelled 5'– or 3'–PTP-oc cDNA probe (B). The numbers indicate the size of DNA markers in kb.

Southern analysis of the PTP-oc gene
To determine the copy number of the PTP-oc gene, the rabbit genomic DNA was digested with BamHI, DraII, KpnI, PvuII, SacI, and the combination of BamHI and HindIII. The digested DNA fragments were subjected to Southern analyses. Both the 5' and 3' probes (as shown in Figure 3) were used for the hybridization. Figure 5(A) shows that multiple bands with different intensities were seen in the blot hybridized with the 5' probe. This may reflect the presence of introns within the 5' region of the gene or of the related genes. Hybridization of the same blot with the 3' probe indicated that a single unique band was obtained in the digestions with BamHI, DraII, KpnI, PvuII and SacI, alone; whereas two bands were evident in the digestion with the combination of BamHI and HindIII (Figure 5B). The simplicity of the DNA restriction patterns while using 3' probe for Southern analysis indicates that the PTP-oc gene exists as a single copy in the rabbit genome.

Evidence that the PTP-oc gene encodes an active PTP
To confirm that PTP-oc indeed encodes a PTP, a bacterial fusion protein (referred to as GST–cPTP-oc) that contained a putative catalytic domain of the PTP-oc linked to the GST protein was produced for analysis of PTP activity. SDS/PAGE analysis revealed that a protein band corresponding to the predicted size (62 kDa) of the GST–cPTP-oc fusion protein was seen in the lysate of IPTG-induced pGEX-cPTP-containing transformants (lane 5 and 6 in Figure 6). The GST–cPTP-oc fusion protein was purified with glutathione–Sepharose affinity chromatography (lane 6 in Figure 6). In addition to the 62 kDa fusion protein, a
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Figure 6 SDS/PAGE analysis of recombinant GST–cPTP-oc

GST–cPTP-oc fusion protein or GST alone was expressed and purified as described in the Experimental section. The purified proteins were then electrophoresed on a 10% SDS/PAGE and stained with Coomassie Blue. Molecular sizes are indicated in kDa. Lanes 1 and 4, 10 µl of bacterial lysate before IPTG induction; lanes 2 and 5, 10 µl of IPTG-induced bacterial lysate; lanes 3 and 6, 5 µl of GST and GST–cPTP-oc purified by glutathione–agarose.

Figure 7 pNPP phosphatase activity of the GST–cPTP-oc fusion protein

GST–cPTP-oc and GST were expressed in E. coli and purified in parallel to near homogeneity by affinity chromatography using glutathione–Sepharose (Figure 6). Dephosphorylation of pNPP, expressed as increased absorbance at 410 nm, was measured as a function of time (left-hand panel) and of fusion protein concentration (right-hand panel).

Few unexpected smaller proteins were present with the purified fusion protein preparations, presumably resulting from autocleavage of the fusion protein. GST protein was also produced and purified as a control following induction of pGEX-5X-3-containing bacteria by IPTG (lanes 1–3 in Figure 6).

Because previous studies showed that PTPs have significant activities on pNPP, tyrosyl-phosphorylated peptides, and phosphotyrosyl proteins in vitro, and because the physiological substrate of PTP-oc has not been identified, we measured the phosphatase activity of the fusion protein at neutral pH using three artificial substrates: (a) pNPP, (b) tyrosyl-phosphorylated Raytide, and (c) tyrosyl-phosphorylated histone. Figure 7 shows that the GST–cPTP-oc fusion protein was highly efficient in dephosphorylating pNPP, and that the dephosphorylation was proportional to the amount of the fusion protein (right-hand panel) and to the reaction time (left-hand panel). GST alone had no activity on pNPP. The apparent $V_{max}$ and $K_m$ of the enzyme activity with pNPP as the substrate were estimated to be 493 units/mg and 1.37 mM pNPP, respectively. Sodium orthovanadate, a known inhibitor for mammalian PTPs [45], at 50 µM completely inhibited the phosphatase activity of the fusion protein (results not shown). The apparent $K_i$ for vanadate was estimated to be 0.51 µM. Similarly, Figure 8 indicates that the GST–cPTP-oc, but not the GST, was effective at dephosphorylating the $[^{32}P]$tyrosyl-phosphorylated Raytide in a time- (left-hand panel) and dose-dependent (right-hand panel) manner. Figure 9 shows that while PTP fusion protein exhibited strong phosphatase activity toward $[^{32}P]$tyrosyl-phosphorylated histone, it was ineffective in dephosphorylating $[^{32}P]$seryl-phosphorylated casein under the same conditions.

DISCUSSION

In this study, we have cloned and completely sequenced a putative full-length PTP (i.e. PTP-oc) cDNA from a rabbit osteoclast cDNA library. This gene indeed encodes a PTP because the GST–cPTP-oc fusion protein displayed strong activity toward pNPP, tyrosyl-phosphorylated Raytide and histone at neutral pH. It is a phosphotyrosine-specific phosphatase and not a dual-specificity phosphatase since the PTP-oc fusion protein was unable to dephosphorylate phosphoseryl casein under the same...
conditions. The PTP catalytic domain of PTP-oc showed a relatively low level of sequence identity with that of the known PTPs. This low level of identity is unlikely to be attributed to the species variance, since the species difference usually accounts for less than 10% sequence variance among PTP homologues. For instance, human RPTPγ and its murine homologue are 95% identical in amino acids with 90% identity in nucleotide sequence [24].

There is circumstantial evidence supporting the possibility that PTP-oc is a member of the transmembrane PTP family. Accordingly, PTP-oc contains a stretch of hydrophobic amino acid residues, followed by several highly charged residues, at a region proximal to the N-terminus. These structural properties are well known characteristics of a transmembrane domain. On the other hand, the lack of a signal peptide and a large extracellular domain are incompatible with the premise that PTP-oc is a transmembrane protein. In addition, the C-termini of some cytosolic PTPs (e.g. PTP-1B and T-cell PTP) have been shown to contain a hydrophobic domain, which serves as an interacting domain with endoplasmic reticulum [46]. (Activation of these cytosolic PTPs requires the removal of the hydrophobic C-termini [39,47].) Thus, we cannot entirely rule out the possibility that PTP-oc is a cytosolic enzyme, which has a regulatory, hydrophobic domain at the N-terminus. However, we favour the possibility that PTP-oc is a ‘transmembrane’ PTP because the catalytic domain of PTP-oc shares a higher degree of sequence similarity with that of transmembrane PTPs than that with cytosolic PTPs. Future immunocytochemical studies are required to determine its subcellular localization within the osteoclast.

There are three noteworthy observations regarding the molecular structure of PTP-oc when compared with that of other mammalian PTPs (Figure 10): first, PTP-oc is probably one of the smallest ‘transmembrane’ PTPs, since it is much smaller than most membrane PTPs, whose size is in the range of 80000 to 250000 Da [18,23]. Secondly, unlike most known transmembrane PTPs, the putative intracellular portion of the PTP-oc contains only a single catalytic domain rather than two tandem repeats. Thirdly, in contrast to most transmembrane PTPs whose extracellular domain is relatively large (several hundred residues in length) and contains multiple glycosylation sites, Ig-like and/or FN-III domains, or carbonic anhydrase-like domains [23,24,27,44], the extracellular domain of PTP-oc is very short and lacks these domains. Taken together, these distinct molecular features suggest that PTP-oc may represent a new subfamily of transmembrane PTPs.

The expression of the PTP-oc mRNA appeared to be restricted to the osteoclasts, the spleen, the kidney and the brain. However, it is intriguing to note that the kidney and brain (but not spleen and osteoclasts) expressed, in addition to the 3.8 kb PTP-oc mRNA transcript, also a larger 6.5 kb related mRNA transcript. It has been suggested that mouse osteoclast progenitor cells are derived from the mouse spleen haematopoietic stem cells [48]. Accordingly, that the PTP-oc mRNA is present in the spleen and osteoclasts may suggest that the expression of the PTP-oc (and not the 6.5 kb transcript) is unique to osteoclasts and precursor cells.

The 6.5 kb transcript, referred to as GLEPP1, has recently been cloned from a rabbit kidney cDNA library [49]. Comparison of the nucleotide and predicted amino acid sequences of the kidney GLEPP1 with those of the PTP-oc revealed that the intracellular domains of these two isoenzymes shared approx. 90% sequence identity, indicating that these two isoenzymes are related. However, the kidney enzyme contains a large extracellular domain, comprising eight repeats of an FN-III-like motif and 15 putative N-glycosylation sites, whereas PTP-oc contains only a very short (i.e. eight residues) putative extracellular domain. Most importantly, PTP-oc contains a unique region of 28 amino acids (residues 66–93 of Figure 1, shown as bold and italic letters) inserted in the intracellular domain, which is absent in the kidney isoenzyme. Thus, PTP-oc appears to be a truncated version of the kidney GLEPP1, presumably resulting from an alternative splicing at the 5’ end of the gene. Accordingly, PTP-oc may be a splicing variant of the kidney isoenzyme. The presence of a splicing variant in osteoclasts and precursor cells (i.e. spleen cells) might play a tissue-specific regulatory role. In this regard, it has been shown that alternative splicing plays a key role in producing alternative variant transcripts for transmembrane PTPs, including human CD45/LCA [50] and rat PTP-P1/PTP-PS [51].

The physiological relevance of a short ‘extracellular’ domain is unclear. The short extracellular domain does not appear to be unique to PTP-oc since the extracellular domain of HPTPε is also very short, i.e. 27 amino acid residues in length [23]. It has been suggested that the extracellular domains, especially the Ig-like and FN-III repeats, of transmembrane PTPs may function as cell-surface receptors or specific binding sites for ligands [23,27,44]. Consistent with this speculation are the findings that Ig-like and FN-III repeats are found at the ligand binding sites of neural cell adhesion receptors [52,53]. Accordingly, it is unlikely that the short extracellular domains of PTP-oc and HPTPε function as cell-surface receptors. However, we cannot overlook the possibility that the extracellular domain of PTP-oc (and HPTPε) could be part of a multi-subunit receptor complex forming between PTP-oc and one or more molecules with large extracellular regions. Alternatively, because the 33 N-terminal amino acid residues (i.e. the eight residues of the extracellular domain and the 25 residues of the transmembrane domain) of the PTP-oc could function as a weak signal for mitochondria-association according to the prediction by the TRANSPEP program of PC GENE, it may be speculated that PTP-oc could be a mitochondria-associated protein in which the N-terminus may serve as an attaching site with the mitochondrial membrane. Additional work is needed to address these possibilities.

While we are beginning to understand the molecular structure of this unique osteoclastic PTP-oc, its physiological significance and regulation are unknown. However, the expression of PTP-oc mRNA occurred primarily in osteoclasts, suggesting that this
PTP-oc could play a functional role in regulating tyrosyl phosphorylation levels in osteoclasts. Because protein tyrosyl phosphorylation mediated by pp60src PTK has been shown to be absolutely essential for osteoclast activity [6–8], we speculate that this PTP-oc might be involved in the regulation of the protein tyrosyl phosphorylation either by countering the effects of the PTKs (e.g. pp60src) through the specific dephosphorylation of the cellular substrates of PTKs, or by altering the PTK activity of pp60src through specific dephosphorylation of this PTK. (The PTK activity of pp60src is inhibited by phosphorylation at Tyr327 [54]). Supporting the latter speculation is the recent demonstration that the pp60src dephosphorylation and activation can be mediated by the receptor-like transmembrane PTP [55]. Nevertheless, it seems likely that the physiological role of this PTP-oc will remain undetermined until its physiological substrate(s) is identified.

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