# Biochemical Characterization of Osteo-Testicular Protein Tyrosine Phosphatase and Its Functional Significance in Rat Primary Osteoblasts

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ABSTRACT: Rat osteo-testicular protein tyrosine phosphatase (OST-PTP), expressed in osteoblasts and testis, is a receptor-like transmembrane protein with two tandemly repeated phosphatase domains in the cytoplasmic region. In this report, we show that the first domain (CD1) is enzymatically active and appears to be influenced by the catalytically inactive second domain (CD2). The activity of CD1 is specific to phosphorylated tyrosine. Full-length OST-PTP protein expressed in COS cells has a molecular mass of approximately 185 kDa, and immunoprecipitates of this protein using OST-PTP-specific antisera show strong tyrosine phosphatase activity. Expression of OST-PTP mRNA in primary rat calvarial osteoblasts is temporally regulated, and peak expression is found at approximately day 15, which correlated well with the appearance of OST-PTP protein and its associated tyrosine phosphatase activity. Treatment of osteoblasts in culture with antisense oligonucleotides directed against the 5′ untranslated region of OST-PTP results in abrogation of differentiation, confirming the functional importance of OST-PTP expression in osteoblast development.

Protein tyrosine phosphatases (PTPases)[1] play an important role in the regulation of cell proliferation, differentiation, transformation, and cytoskeletal integrity in response to numerous external stimuli (*1*−*3*). The level of tyrosine phosphorylation on substrate proteins in a given cell is balanced by the opposing actions of protein kinases and PTPases (*4*). A number of PTPases have been identified and are now known to belong to a multimember family comprised of nonreceptor cytoplasmic enzymes and receptor-like transmembrane molecules (*3*, *5*, *6*). Receptor-like PTPases have common structural features that include an extracellular domain of varying length and composition, a single membrane spanning region, and one or two intracellular catalytic domains *(7, 8)*. The active catalytic domain possesses a consensus signature motif [I/VHCxAGxxR(S/T)G where x = any amino acid] and bears no resemblance to that of serine/threonine, alkaline, or acid phosphatases (*6*). Receptor-like PTPases represent a novel mechanism of extra- to intracellular signal transmission via modulation of tyrosine phosphorylation status. For example, the receptor type PTPase, PTPu, has been shown to exhibit homophilic interactions mediated solely by the extracellular domain and potentially offers a direct link between cell adhesion events and the triggering of signal transduction pathways (*9*, *10*).

Regulation of tyrosine phosphorylation appears to be crucial for maintenance of bone tissue as evidenced by recent reports in which disruption of protein tyrosine kinases, particularly c-src or c-fms, leads to impaired bone remodeling resulting in osteopetrosis (*11*, *12*). However, the importance of protein tyrosine phosphatases in bone metabolism is limited. Recently, a novel receptor-like PTPase expressed in bone and testis, named osteo-testicular protein tyrosine phosphatase (OST-PTP), was cloned from a rat osteoblast-like cell line, UMR 106 (*13*). The OST-PTP mRNA was up-regulated following differentiation and matrix formation of primary osteoblasts and down-regulated in actively mineralizing osteoblasts. The cDNA can potentially code for a protein of 1711 amino acids possessing an extracellular domain with 10 fibronectin type III repeats and a cytoplasmic region with 2 catalytic domains that has 45% sequence identity to the human PTP$\beta$. Catalytic domain 1 has the characteristic active site of PTPases while domain 2 contains a divergent active site but possesses many of the surrounding conserved motifs (*13*). It has been shown that the cytoplasmic region containing the two domains exhibits tyrosine phosphatase activity (*13*). Mauro et al. (*13*) have also shown that OST-PTP mRNA is up-regulated by parathyroid hormone in the UMR 106 cell line and it is expressed in a stage-specific manner in seminiferous tubules of rat testis. Nevertheless, the significance of the two catalytic domains and the role of OST-PTP in osteoblast differentiation are not known. In the present paper, we describe (a) the effect of the CD2 domain on the phosphatase activity of the CD1 domain of OST-PTP; (b) the phosphatase activity of full-length OST-PTP; (c) the identification of the native protein in primary rat osteoblast cells; (d) the expression profile of OST-PTP protein and activity during osteoblast differentiation in vitro; and (e) the functional importance of OST-PTP protein in mineralization and nodule formation in rat osteoblast cultures using anti-sense oligonucleotides.

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[1] Abbreviations: OST-PTP, osteo-testicular protein tyrosine phosphatase; PTPase, protein tyrosine phosphatase; EC, extracellular domain; CD1, catalytic domain 1; CD2, catalytic domain 2; IC, intracellular domain.

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## EXPERIMENTAL PROCEDURES

*Expression of OST-PTP Domains in E. coli*. Different regions of rat OST-PTP cDNA, extracellular domain (EC), catalytic domain 1 (CD1), catalytic domain 2 (CD2), and the intracellular domain containing both CD1 and CD2 (IC) were cloned into the *Nde*I and *Sal*I sites of the pET 23b vector (Novagen, Madison, WI) (Figure 1). In this vector, the insert is transcribed from a T7 promoter, and the resulting protein has six histidine residues at the C-terminus. For protein expression, the various constructs were transformed into the BL21 (DE3) strain of *E. coli* and induced with IPTG. The expressed proteins were purified using His-Bind resin column chromatography (Novagen), and EC and IC proteins were used to raise antibodies in goats. EC and IC immune sera were affinity purified using the respective antigens coupled to Sepharose matrixes.

*Site-Directed Mutagenesis*. Site-directed mutagenesis of OST-PTP cDNA to produce C1350S and H1349P mutants was carried out using oligonucleotide-directed mutagenesis (*14*). Specific nucleotide changes were confirmed by DNA sequencing.

*Expression of OST-PTP in COS Cells*. Full-length OST-PTP cDNA and mutated cDNAs were cloned into pcDNAIII. COS cells were transfected with OST-PTP cDNA plasmids in the presence of Transfectam reagent (Promega, Madison, WI) using the manufacturer's procedure. Forty-eight hours later, the cells were harvested for characterization of the expressed protein.

*Enzyme Assays*. Kinetic analysis of the recombinant protein was done using hydrolysis of *p*-nitrophenyl phosphate (pNPP) as a measure of phosphatase activity according to standard assay conditions (*15*). Michaelis−Menten analysis of the data was performed using Sigma plot. Tyrosine-phosphorylated Raytide, a standard peptide substrate for tyrosine phosphatases, and serine-phosphorylated Kemptide, a standard peptide substrate for Ser/Thr phosphatases, were used to determine substrate specificity using a kit from Promega.

*Metabolic Labeling and Immunoprecipitation*. Transfected cells were washed with serum-free medium lacking methionine and cysteine, and then incubated for 6 h in the presence of [$^{35}$S]methionine/cysteine (New England Nuclear, Boston, MA). The cells were then harvested; lysates were prepared in RIPA buffer and immunoprecipitated according Ng et al. (*16*). Briefly, equal amounts of cell lysates were incubated with preimmune serum (P) or immune serum against the extracellular domain (EC) or the intracellular domain (IC) of OST-PTP, and the resulting immune complexes were captured using Protein A-Sepharose (Pharmacia, Piscataway, NJ) beads. Proteins bound to the beads were separated on a 6% SDS−PAGE gel and autoradiographed.

*Phosphatase Assay*. Transfected COS cells or primary osteoblast cells were harvested in lysis buffer (20 mM Tris, pH 7.5, containing 0.5% Triton X-100, 150 mM NaCl, 2.0 mM EDTA, 0.2 mM PMSF, 1.0 $\mu$g/mL pepstatin, 1.0 $\mu$g/mL leupeptin, and 1.0 $\mu$g/mL aprotinin) and incubated on ice for 10 min as described by Ng et al. (*16*). Control lysates were prepared from mock-transfected COS cells. Cell lysates were clarified by centrifugation and the supernatants precleared with Separose CL-4B (Pharmacia) at 4 °C for 1 h. Equal amounts of antisera containing antibodies against intracellular and extracellular portions of OST-PTP were added and incubated for 2 h at room temperature. The antigen−antibody complexes were pulled down with Protein-A Sepharose and washed 3 times in lysis buffer and twice in nonreducing PTP buffer (50 mM imidazole chloride, pH 7.2, 1.0 mM EDTA). The beads were then resuspended in a small volume of PTP buffer (50 mM imidazole chloride, pH 7.2, 1 mM EDTA, and 0.1% $\beta$-mercaptoethanol) containing pNPP or phosphorylated peptides. Released inorganic phosphate was measured using a malachite green reagent kit (Promega).

*Assay for Tyrosine Phosphatase Activity*. The substrate specifity of OST-PTP was determined using tyrosine-phosphorylated Raytide (Oncogene Science Inc., Cambridge, MA) and serine-phoshorylated Kemptide (Sigma, St. Louis, MO). Raytide was phosphorylated on tyrosine by incubating 10 $\mu$g of the peptide with 1.2 $\mu$L of [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) in 30 $\mu$L of reaction buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.1 mg/mL bovine serum albumin, 10 mM $MgCl_2$, 0.1 mM ATP, 0.2% $\beta$-mercaptoethanol) and 0.5 $\mu$g of v-src for 30 min at 30 °C. Kemptide was phosphorylated on serine by incubating 10 $\mu$g of the peptide with 3 $\mu$L of [$\gamma$-$^{32}$P]ATP (6000 Ci/mmol) in 50 $\mu$L of reaction buffer (40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP) in the presence of 5 $\mu$g of catalytic subunit of bovine protein kinase A (Fluka, Milwakee, WI) at 30 °C for 30 min. The reactions were terminated by the addition of 10% phosphoric acid and spotted on P81 paper. Free ATP was removed by washing with 0.5% phosphoric acid, and labeled peptide was eluted with 2 × 0.5 mL aliquots of ammonium bicarbonate. The phosphatase acivity of CD-PTP or full-length OST-PTP was determined using 200 $\mu$g of total protein in a 100 $\mu$L assay buffer (Tris-HCl, pH 7.4, 10 000 cpm of phosphorylated substrate) and incubated at 37 °C. Aliquots were removed at specified times, and the remaining phosphorylated peptide was measured by trapping the peptide on P81 filters as described above.

*Primary Osteoblast Culture*. Primary rat osteoblasts were prepared from 21 day old rat fetuses as described by Aronow et al. (*17*). Briefly, minced calvaria were digested with collagenase, and pooled digestion mixtures were plated in 60 mm tissue culture dishes containing modified Eagle's medium (MEM) with 10% fetal bovine serum. When the cells became confluent (typically day 5), the medium was changed to BGJb medium with ascorbic acid and $\beta$-glycerophosphate to induce differentiation that results in nodule formation. The cells were harvested for total RNA isolation and protein labeling on the days indicated. Cells were labeled with [$^{35}$S]methionine/cysteine for 5 h, harvested for immunoprecipitation, and analyzed as described for COS cells. OST-PTP-specific PTPase activity was also measured in the immunoprecipitates from two dishes of unlabeled primary osteoblasts as described for COS cells.

*Quantitation of OST-PTP mRNA*. Total RNA was isolated using RNAzol B reagent (Tel-Test Inc., Friendswood, TX). Ten micrograms of total RNA was resolved on a formaldehyde−agarose gel and transferred to a Magna charge nylon membrane (Micron Separation Inc., West Boroughm, MA). Prehybridization and hybridization were done at 42 °C in 5 × SSC, 1 × Denhardt's solution, 0.1% SDS, 50% formamide, 7.5% dextran sulfate, 0.5% SDS with 100 $\mu$g/mL calf thymus DNA (*18*). Stringent washes were performed at 60

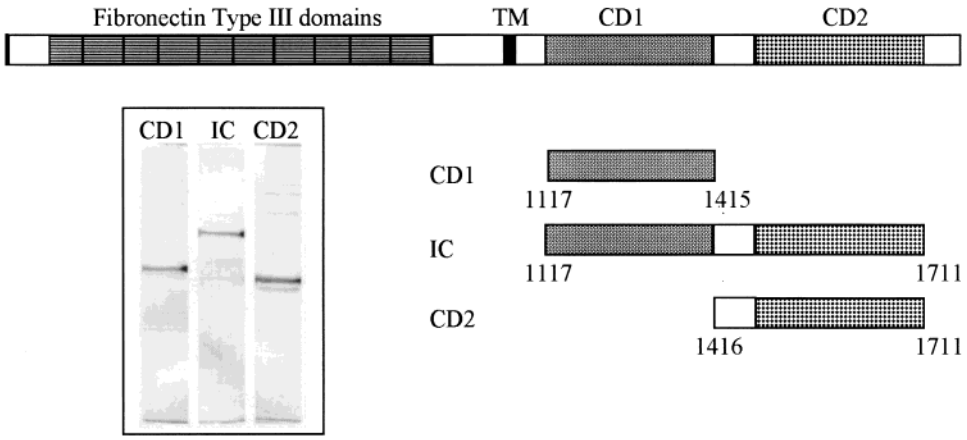


FIGURE 1: Schematic diagram of rat OST-PTP protein: fibronectin type III-like domains; TM, transmembrane domain; catalytic domain 1 (CD1); catalytic domain 2 (CD2); IC, intracellular domain containing CD1 and CD2. CD1, IC, and CD2 were expressed in *E. coli* as described under Experimental Procedures. Numbers refer to the amino acid sequence of OST-PTP. The inset shows a Coomassie blue stained SDS−PAGE gel of purified OST-PTP polypeptides.

°C with 0.2 × SSC/0.1% SDS. Random-labeled DNA probe corresponding to the cytoplasmic domain of OST-PTP (bp 3539−5455) (*13*) was used for hybridization. The blots were reprobed for GAPDH as a control. The hybridization signals were quantified using a scanning densitometer (Molecular Dynamics, Sunnyvale, CA).

*Antisense Treatment*. Phosphorothionate-modified (at the 3′ end) antisense oligonucleotides 18 bases long, directed against the 5′ untranslated regions of OST-PTP, were synthesized and gel-purified. The sequence of antisense OST-PTP oligomer used in the experiment was GATAAAGTCTGGGGACAC. Scrambled oligomer used as negative control had the following sequence: CGCACACGTGTTGGACTAGT. Antisense c-fos oligomer (GCGTTGAAGCCCGAGAAC) was used as a positive control and has previously been shown to successfully inhibit C-fos expression (*19*) and, in turn, specifically inhibit osteoblast differentiation (*20*). The sequences were checked by computer for cross-hybridization with other phosphatase family members and other gene sequences. Beginning on day 1, antisense oligonucleotides were added to osteoblasts in culture once every 24 h in fresh medium containing 10% serum. Antisense oligonucleotides at a concentration of 5 $\mu$M were used, and these doses were found to be nontoxic to the cells (data not shown). Addition of oligonucleotides was continued until day 18, at which point cultures were harvested for counting nodule numbers and measuring secreted osteocalcin levels. On day 13, OST-PTP protein levels were analyzed by labeling the antisense-treated osteoblasts with [$^{35}$S]methionine/cysteine followed by immunoprecipitation with OST-PTP-specific antibodies. The immune complexes were analyzed as described for COS cells.

*Histochemical and Biochemical Analysis*. Osteocalcin levels in primary rat osteoblast culture medium were assayed by radioimmunoassay as described by Gundberg et al. (*21*). Nodule numbers in the culture dishes were counted after staining histochemically with von Kossa reagent (*22*).

## RESULTS

*Characterization of OST-PTP Phosphatase Activity*. There are two tandemly repeated phosphatase domains in the cytoplasmic region of OST-PTP designated as CD1 and CD2. Based on the structural homology with other PTP catalytic sites, CD1 is predicted to be catalytically active because this domain contains essential features of the signature motif of the catalytic site of tyrosine phosphatases. CD2 is predicted to be an inactive domain since CD2 lacks the invariant cysteine residue necessary for catalysis and shares only 42% identity with domain 2 of CD45, a well-studied tyrosine phosphatase (*13*). To determine and characterize the enzymatic activities of CD1 and CD2, proteins containing one or both CDs were expressed in *E. coli* with a histidine tag at the C-terminus (Figure 1). The expressed proteins were purified using nickel column chromatography, and phosphatase activity was measured using pNPP as substrate. As shown in Figure 2A, the first catalytic domain (CD1) was enzymatically active, and significant activity could be detected as early as 15 min. The second domain (CD2) was completely inactive at all concentrations of the substrate tested. CD1 showed optimum pH for enzyme activity at approximately 6.75 with a $K_m$ of 4.5 $\mu$M (data not shown). Interestingly, the intracellular portion (IC) containing both domains showed reduced activity compared to the CD1 alone when tested at equimolar concentrations of the proteins. The presence of the second domain inhibited the activity of the first domain by at least 40% at the peak activity (Figure 2A). When the CD2 domain was co-incubated with the CD1 domain (added in trans), no significant effect was observed on the activity of CD1 (data not shown). This demonstrates that the CD2 domain must lie in close proximity to CD1 to exert inhibitory activity, suggesting that the CD2 domain influences the conformation of the CD1 domain.

To demonstrate substrate specificity of the recombinant protein, purified CD1 was tested for its dephosphorylating activity by monitoring dephosphorylation and release of $^{32}$P from the tyrosine-phosphorylated peptide substrate Raytide and the serine/threonine (Ser/Thr)-phosphorylated peptide substrate Kemptide. As shown in Figure 2B, CD1 was active on Raytide, and completely inactive on Kemptide, at all protein concentrations tested, thus showing that OST-PTP exhibits tyrosine phosphatase activity with no Ser/Thr phosphatase activity. Furthermore, sodium vanadate, a general tyrosine phosphatase inhibitor, virtually abolished dephosphorylation of Raytide by CD1 (data not shown).

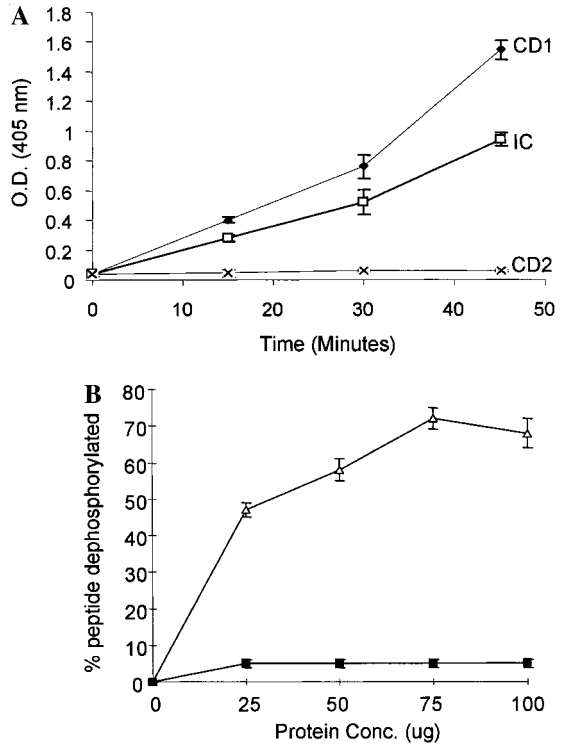


FIGURE 2: Phosphatase activity and substrate specificity of recombinant OST-PTP: CD1, CD2, and IC-PTP were expressed as fusion proteins in *E. coli*. Phosphatase activity was performed using pNPP as substrate. (A) Comparison of phosphatase activities. Equimolar concentrations of purified proteins were assayed for phosphatase activity. Activity is expressed as optical density (OD) units measured at 405 nm. (B) Assessment of substrate specificity. The ability of CD1 to dephosphorylate tyrosine-phosphorylated Raytide (△) and serine-phosphorylated Kemptide (■) was determined at various protein concentrations.

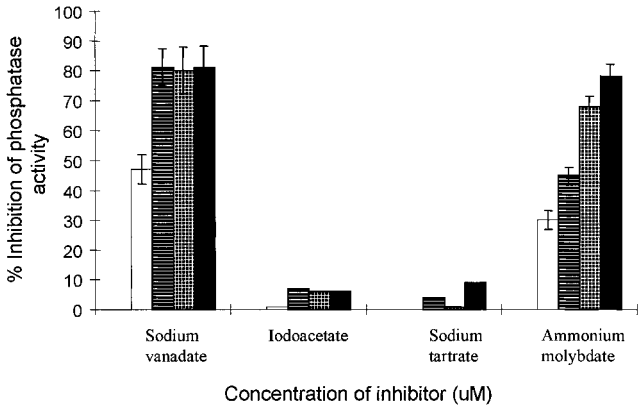


FIGURE 3: Testing of various known phosphatase inhibitors on CD1-PTP phosphatase activity. The activity of 10 $\mu$g of CD1 protein was determined in the presence of increasing concentrations of known phosphatase inhibitors in the pNPP assay, and the results are expressed as percentage inhibition of phosphatase activity. Sodium vanadate, iodoacetate, ammonium molybdate, and sodium tartrate were tested at 0.0625 (□), 0.125 (striped bars), 1.25 (stippled bars), and 12.5 $\mu$M (■) concentrations.

To further evaluate the specificity of OST-PTP phosphatase activity, the ability of known phosphatase inhibitors to inhibit CD1 activity was assessed (Figure 3). Sodium vanadate and ammonium molybdate, two tyrosine phosphatase inhibitors, were potent inhibitors of CD1 activity. In contrast, sodium tartrate and iodoacetate, inhibitors of Ser/Thr phosphatases, did not inhibit the activity of CD1. The activities of these various phosphatase inhibitors further demonstrate that OST-PTP is a tyrosine phosphatase and not a Ser/Thr phosphatase.

*Expression and Activity of Full-Length OST-PTP in COS Cells*. To assess the biochemical and enzymatic properties of full-length OST-PTP, wild-type and mutant OST-PTP proteins containing mutations in the predicted active site of the CD1 region (C1350S and H1349P) were expressed in COS cells. Cells were metabolically labeled, and lysed extracts were subjected to immunoprecipitation with antiserum recognizing either the extracellular (EC) or the intracellular (IC) domain. As shown in Figure 4A, both antisera precipitated a single protein with a molecular mass of approximately 185 kDa, consistent with the predicted size of OST-PTP. Similar expression levels were obtained with the three OST-PTP plasmids, and the wild-type and mutant OST-PTPs are recognized by both antisera.

The tyrosine phosphatase activity of full-length OST-PTP was determined following transfection and expression in COS cells. Transfected cell extracts were immunoprecipitated with OST-PTP-specific antisera, and the phosphatase activity of the immunoprecipitate was measured. Wild-type protein showed strong catalytic activity (Figure 4B). Notably, the EC and IC antisera did not inactivate the enzyme, which therefore suggests that antigenic epitopes recognized by these antisera do not include the active site. More importantly, proteins containing mutations in the predicted catalytic site were completely inactive. Furthermore, OST-PTP protein expressed in COS cells dephosphorylated Raytide (Figure 4C) and was completely inactive on Kemptide, (data not shown), consistent with the activity of the CD1 protein in *E. coli* (Figure 2B). Mutant proteins did not dephosphorylate either of the two substrates (data not shown). The dephosphorylation of Raytide was inhibited by sodium vanadate, again consistent with the activity of the CD1 protein (Figure 3).

*Identification of OST-PTP in Primary Rat Osteoblasts*. OST-PTP was originally cloned from UMR 106 cells, a rat osteoblast cell line. In an earlier paper, Mauro et al. (*13*) analyzed OST-PTP mRNA expression at three time points during differentiation of primary rat osteoblasts and observed regulation of the message during differentiation. We have extended this observation to include the expression profile of OST-PTP mRNA during osteoblast growth and development in primary rat osteoblast cultures. As shown in Figure 5A, the levels of OST-PTP mRNA are undetectable until day 7 in culture and begin to increase thereafter, reaching peak levels by day 15. The mRNA levels then decline to low levels and reach a plateau.

The expression of OST-PTP protein during osteoblast development was also determined. Primary rat osteoblast cells cultured for 5, 12, and 27 days were labeled with [$^{35}$S]-methionine/cysteine and immunoprecipitated with a mixture of EC antiserum and IC antiserum. As can be seen in Figure 5B, OST-PTP antisera specifically immunoprecipitated a protein of ~190 kDa. Densitometric scans of the protein band demonstrated very low levels of the protein in day 5 cultures, increasing by greater than 5-fold in day 12 cultures, and undetectable levels in day 27 cultures. The presence of a very low level of OST-PTP protein on day 5 suggests that a low level of OST-PTP mRNA is present at day 5, but is not

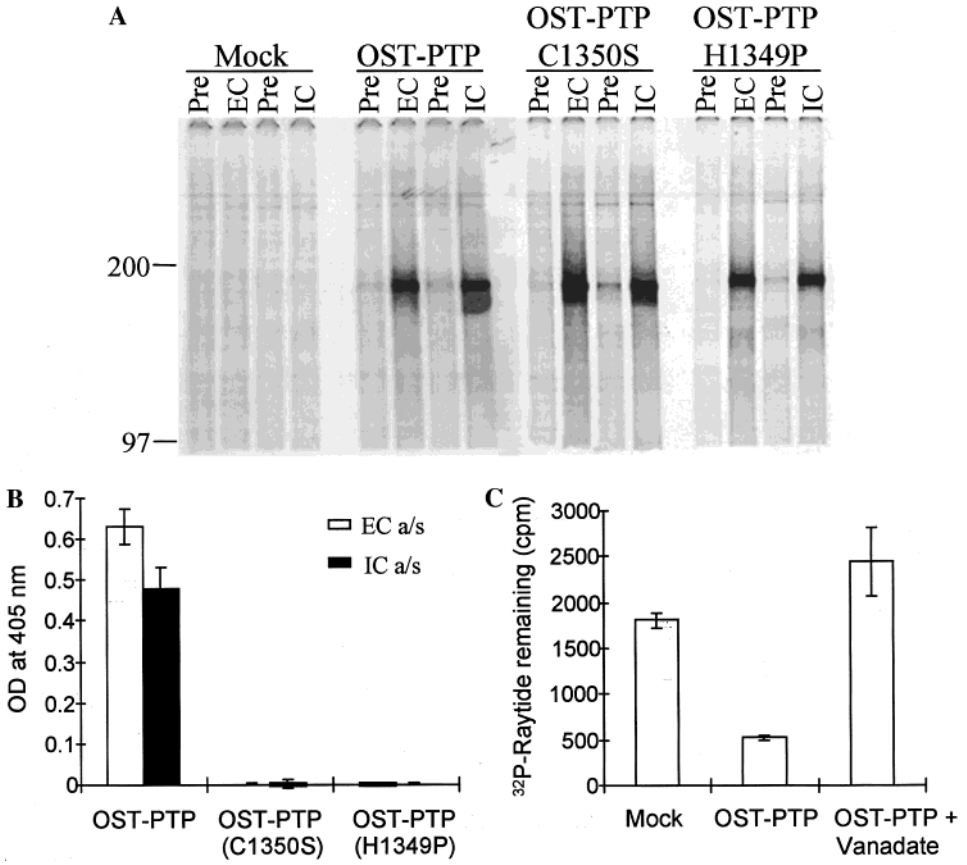


FIGURE 4: Characterization of OST-PTP protein expressed in COS cells. COS cells were transfected with cDNA plasmids [mock (no insert), OST-PTP, mutants C1350S and H1349P] using Transfectam reagent and incubated for 48 h. (A) SDS−PAGE analysis of labeled COS cell lysates immunoprecipitated with OST-PTP antisera. Equal amounts of cells lysates were incubated with preimmune serum (Pre) or immune serum against the extracellular domain (EC) or the intracellular domain (IC) of OST-PTP, and the immunoprecipitates were analyzed on a 6% SDS−PAGE gel. (B) OST-PTP-specific phosphatase activity in transfected COS cells: OST-PTP protein was immunoprecipitated with either EC or IC antiserum, and the associated phosphatase activity was measured using pNPP as substrate. (C) OST-PTP-specific tyrosine phosphatase activity: COS cells transfected with native OST-PTP cDNA were harvested and incubated with a mixture of equal amounts of EC and IC antisera. The immunoprecipitates were tested for their ability to dephosphorylate $^{32}$P-labeled Raytide in the presence and absence of 5 mM sodium vanadate. Remaining phorphorylated peptide was measured as described under Experimental Procedures.

detectable by Northern blot using total RNA (Figure 5A). Immunoprecipitates from these three cultures were then assayed for phosphatase. As shown in Figure 5C, enzyme activity was observed and the increase in activity correlated with the increase in OST-PTP protein levels, with the highest levels observed during the differentiation phase of the cultures.

*Inhibition of OST-PTP Expression in Primary Osteoblast Cultures*. The time course of OST-PTP expression in rat osteoblast cultures suggests that OST-PTP may play an active role in osteoblast differentiation. To examine the role of OST-PTP in osteoblast differentiation, we used antisense oligonucleotides to inhibit the expression of OST-PTP protein and determine the consequences of this inhibition on the levels of osteocalcin in the medium and the number of nodules formed, two markers of differentiation. Primary osteoblast cultures were treated from day 1 through day 18 with antisense OST-PTP oligomers directed against the 5′ end of OST-PTP messenger RNA, and the cultures were harvested on day 18. To examine the effect of antisense oligmers on OST-PTP protein expression, osteoblasts that were treated with either scrambled or antisense OST-PTP oligomers were labeled with [$^{35}$S]methionine/cysteine on day 13 (around which maximum levels of OST-PTP protein were found in osteoblasts), and cell lysates were immunoprecipitated with anti OST-PTP antibody. As shown in Figure 6A, SDS−PAGE analysis of immunoprecipitates clearly demonstrates that there is a drastic reduction in OST-PTP protein level in OST-PTP antisense oligmer-treated osteoblasts compared to the ones that received only scrambled oligomer. In additon, the antisense oligonucleotide was very effective in blocking osteoblast differentiation as shown by an 80% reduction in the number of nodules compared to controls (Figure 6B). This reduction was comparable to the 72% reduction observed with antisense c-fos oligomer tested in the same experiment. The scrambled oligomer control had little to no effect on the number of nodules. Osteocalcin levels in the culture medium showed a parallel drop in antisense OST-PTP-treated and antisense c-fos-treated cultures (Figure 6B). In contrast, control cultures receiving the scrambled oligomers did not show any significant changes in osteocalcin levels. Furthermore, cell proliferation was not affected by antisense oligomer treatment since treated and control cultures had similar abilities to convert tetrazolium salt into a formazan product in a cytotoxicity assay (Promega), an indirect measure of cell number and cell viability (data not shown). Therefore, the inhibition of nodule formation and osteocalcin production by an antisense oligonucleotide to

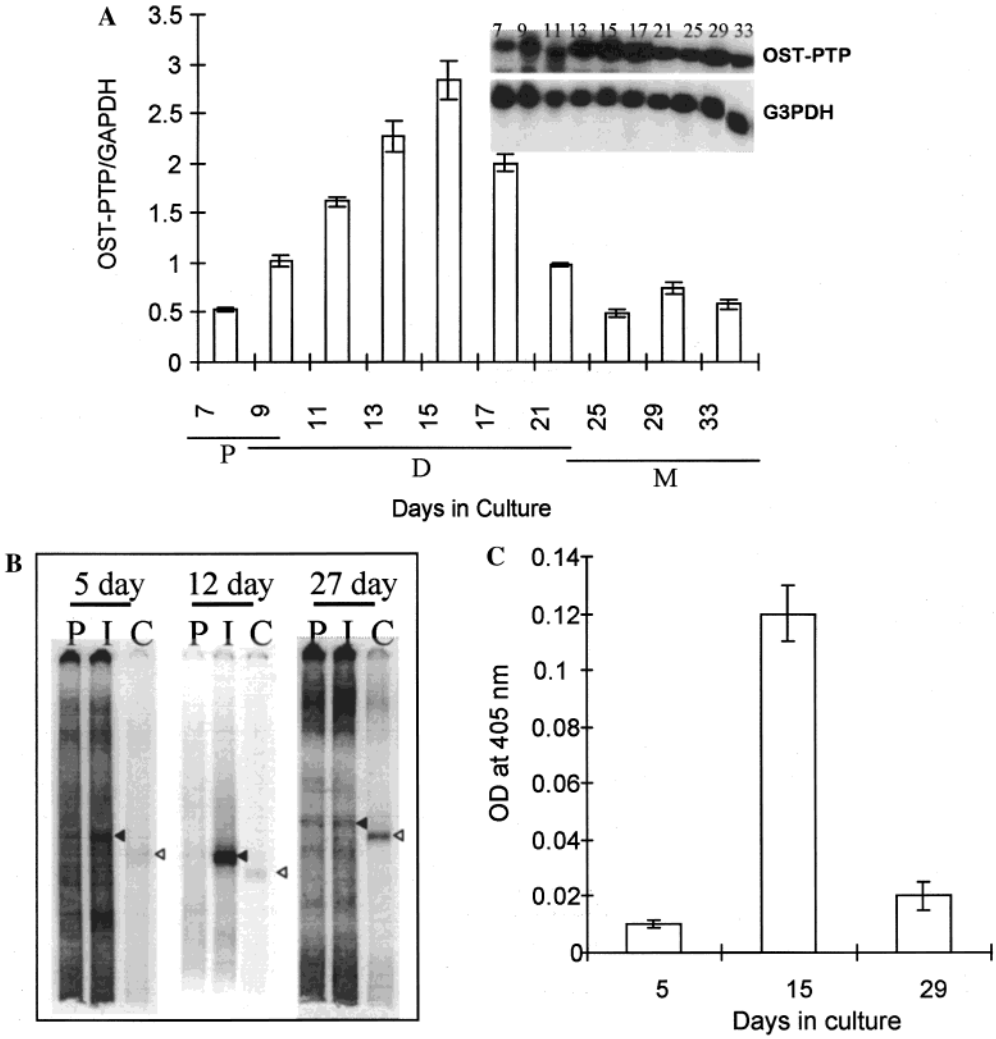


FIGURE 5: Differential expression of the OST-PTP gene in fetal rat calvaria-derived primary osteoblast cultures. Osteoblasts derived from 21 day old rat fetal calvarial were cultured in the presence of ascorbic acid (50 $\mu$g/mL) and $\beta$-glycerol phosphate (10 mM). (A) OST-PTP mRNA analysis: Total RNAs from varying day old cultures were prepared, and OST-PTP and GAPDH mRNAs were quantified by hybridizing 10 $\mu$g of total RNA to the respective probes on a Northern blot. Results are expressed as the ratio of OST-PTP- and G3PDH-specific signals. Developmental stages of osteoblast cultures are indicated as P for proliferation, D for differentiation, and M for mineralization. The inset shows the Northern blot autoradiogram. (B) SDS−PAGE analysis of OST-PTP immunoprecipitates prepared from osteoblasts at different stages of growth and differentiation: proliferation (day 5), maturation (day 12), and mineralization (day 27). Osteoblasts were labeled with [$^{35}$S]methionine/cysteine, and the lysates were incubated with either preimmune sera (P) or a mixture (1:1) of immune sera against EC and IC portions of OST-PTP (I). $^{35}$S-labeled OST-PTP expressed in COS cells and immunoprecipitated with OST-PTP antisera was included as a control (C). The open arrowheads show the migration of OST-PTP protein expressed in COS cells at 185 kDa. The closed arrowheads show the migration of native OST-PTP protein from osteoblasts at 190 kDa. (C) OST-PTP-associated phosphatase activity in primary osteoblasts: 5, 15, and 29 day old osteoblast cultures were harvested and immunoprecipitated with a mixture (1:1) of immune sera against the EC and IC portions of OST-PTP, and phosphatase activity was measured using pNPP as substrate.

OST-PTP suggests that OST-PTP plays an active role in osteoblast differentiation.

## DISCUSSION

During osteoblast growth and differentiation in culture, there is an ordered expression of genes that allows the process to be divided into three stages: proliferation, maturation, and mineralization (*17*). Rat fetal calvaria derived osteoblasts show very active proliferation in culture during the first few days and are associated with high expression of cell cycle and cell growth regulated genes. This is followed by a maturation stage in which the cells express proteins associated with bone cell phenotype, including alkaline phosphatase, osteopontin, and osteocalcin. The cells then progress into a mineralization stage expressing maximum levels of bone-specific genes, deposition of minerals, and the resulting formation of nodules (*23*). In heavily mineralized cultures, apoptotic cells are detected, suggesting programed cell death and a mechanism by which further differentiation of osteoblasts into osteocytes is regulated (*24*). This temporal and stringently regulated progression of osteoblast growth and differentiation in culture can be modified by various hormones and growth factors, and the responsiveness of a particular gene is a function of the developmental stage of the osteoblast (*25*, *26*).

The mRNA of OST-PTP, a novel receptor-like protein tyrosine phosphatase expressed in bone and testis, was reported to be up-regulated in differentiating cultures of osteoblasts and down-regulated in late stage mineralizing cultures (*13*). As demonstrated here, the CD1 domain of OST-PTP, the first of the two catalytic domains, was enzymatically active and specifically dephosphorylated ty-

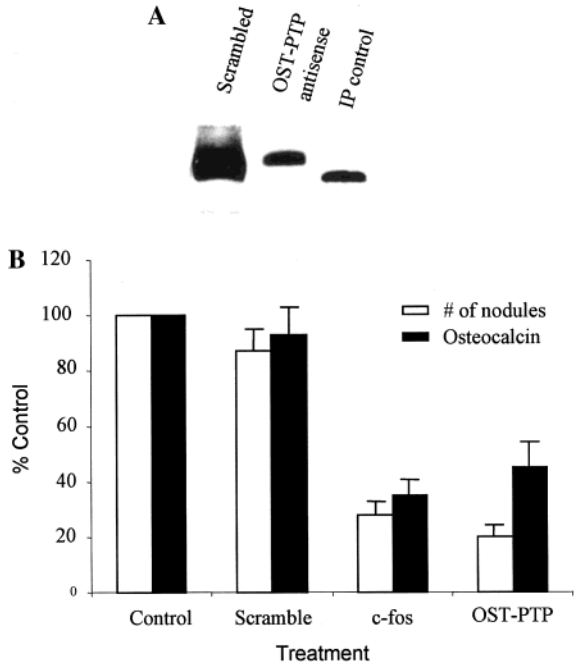


FIGURE 6: Effect of antisense oligonucleotides on nodule formation and osteocalcin expression in primary rat osteoblast cultures. Primary rat osteoblast cultures were treated with either c-fos, anti-OST-PTP, or scrambled oligonucleotides at a concentration of 5 $\mu$M from day 1 to day 18. (A) Autoradiogram of OST-PTP immunoprecipitates prepared from [$^{35}$S]methionine/cysteine-labeled osteoblasts that were treated with either scrambled or antisense OST-PTP oligomers from day 1 to day 13. IP control is the immunoprecipitate prepared from labeled OST-PTP-transfected COS cells. (B) Osteocalcin levels and nodule numbers were determined on day 18 and expressed as a percentage control.

rosine-phosphorylated substrates, an activity which was inhibited by sodium vanadate, a specific inhibitor of protein tyrosine phosphatases. The second domain, CD2, was inactive and appeared to regulate the catalytic activity of the first domain only when it was expressed in the cis configuration. In the case of T cell-PTP, there is evidence to suggest a role of noncatalytic C-terminal sequences in the regulation of enzyme activity (*5*). Furthermore, mutational analysis of CD45 indicated that there are interactions between the two catalytic domains, in that a mutation in domain 2 appears to alter the specificity of domain 1 (*27*, *28*). The data presented here suggest that a similar scenario may apply to OST-PTP.

In the case of LAR, both catalytic domains 1 and 2 contain the signature sequence necessary for the phosphatase activity, but only domain 1 is enzymatically active. By analyzing the crystal structure of a fragment of LAR that includes domain 1 and domain 2 (residues 1308−1881), Nam et al. (*7*) concluded that though the active site topologies within the two domains are very similar to each other, the structural differences resulting from the substitution of two highly conserved residues (Leu 1644 and Glu 1779 in D2) in domain 2 are responsible for the lack of activity. In addition, the authors suggested that domain 2 may be an active moiety in the correct cellular context and either modification of the protein or change in cellular environment might activate this domain in vivo. In other PTPs, particularly in the case of T cell-PTP and PTP1B, there are reports suggesting that the noncatalytic C-terminal sequences direct both enzymes to membranes (*5*).

Full-length OST-PTP cDNA expressed in COS cells encoded a 185 kDa protein that was immunoprecipitated with specific antiserum. The immunoprecipitated protein had tyrosine phosphatase activity, and this activity was abolished by mutating a critical cysteine residue in the first catalytic domain. Interestingly, the size of the recombinant OST-PTP protein expressed in COS cells was somewhat smaller than the endogenous OST-PTP protein present in osteoblast cultures from rat calvaria. Differences in posttranslational modifications between the two cell types may account for the differences in molecular size. Indeed, there are 16 potential N-glycosylation sites scattered throughout the extracellular portion of OST-PTP that may be differentially glycosylated within different cell types (*13*).

In primary rat calvarial osteoblast cultures, the expression profile of OST-PTP mRNA indicates that this gene is expressed at the transition from the proliferation stage to the maturation stage of osteoblasts. The levels of OST-PTP protein and the associated phosphatase activity also showed a similar pattern, suggesting an important role for OST-PTP in this transition. We therefore studied the functional importance of OST-PTP in osteoblast maturation by inhibiting the expression of OST-PTP protein using OST-PTP-specific antisense oligonucleotides. Indeed, suppression of OST-PTP protein production during the growth period resulted in a reduction of osteocalcin levels, a marker of mature osteoblast phenotype, and a subsequent reduction in nodule formation in the mineralization stages of the culture. These effects were similar to those observed with c-fos antisense oligomers shown here and previously reported (*20*) and therefore suggest that normal OST-PTP expression, like c-fos, is critical for osteoblast function and therefore bone formation. The pattern of expression of OST-PTP during osteoblast maturation might suggest that the protein is involved in signaling the cells to enter into the differentiation stage. Indeed, the structural features of OST-PTP, including an extracellular domain with structural similarity to cellular adhesion molecules and an intracellular domain with phosphatase activity, are well suited for such signaling purposes.

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