Expression, purification, and characterization of human osteoclastic protein-tyrosine phosphatase catalytic domain in *Escherichia coli* ≺

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A B S T R A C T

Osteoclastic protein tyrosine phosphatase (PTP-oc) is a structurally unique transmembrane protein tyrosine phosphatase (PTP) that contains only a relatively small intracellular PTP catalytic domain, does not have an extracellular domain, and lacks a signal peptide proximal to the NH2 terminus. The present study reports the expression, purification, and characterization of the intracellular catalytic domain of PTP-oc (ΔPTP-oc). ΔPTP-oc was expressed in *Escherichia coli* cells as a fusion with a six-histidine tag and was purified via nickel affinity chromatography. When with para-nitrophenylphosphate (p-NPP) as a substrate, ΔPTP-oc exhibited classical Michaelis–Menten kinetics. Its responses to temperature and ionic strength were similar to those of other PTPs. The optimal pH value of ΔPTP-oc is approximately 7.0, unlike other PTPs, whose optimal pH values are approximately 5.0.

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Introduction

Intracellular tyrosyl-phosphorylation levels are tightly regulated by protein tyrosine kinases (PTKs) 1 and protein tyrosine phosphatases (PTPs) [1]. In this process, the phosphorylation reaction is mediated by PTKs, whereas the dephosphorylation reaction is catalyzed by PTPs [1,2]. The importance of tyrosine phosphorylation is illustrated best in osteoclasts [6,7], when considering the major signaling pathways, such as those mediated by RANKL and M-CSF [8,9]. Molecular studies indicate that phosphorylation of proteins on tyrosine residues is critical for the production and function of osteoclasts [2–5]. However, beyond these roles, compelling evidence also supports protein tyrosine phosphorylation having essential regulatory functions in integrin signaling in osteoclasts [10–12].

Osteoclastic protein tyrosine phosphatase (PTP-oc) belongs to the receptor PTP family and is mainly expressed in precursors of osteoclasts, including B lymphocytes, cells of the monocyte-macrophage lineage, and mature osteoclasts; it is also an activator of osteoclast differentiation and activity [13–16,20,21]. PTP-oc is the product of a gene assigned to human chromosome 12p12-p13 [17,18] or mouse chromosome 6 [19], and the expression of PTP-oc is driven by an intronic, tissue-specific proximal promoter of the PTPRO gene [13,14]. Unlike conventional transmembrane PTPs, PTP-oc is constitutive and consists of only 405 amino acid residues, which is relatively small for a transmembrane protein. The structure of PTP-oc is unique in that it contains only a single intracellular PTP catalytic domain, has a very short extracellular domain and lacks a signal peptide proximal to the NH2 terminus [13,16].

PTP-oc is an interesting protein tyrosine phosphatase, as studies show that it not only displays tissue-specific expression but also has tissue-specific functions [14]. PTP-oc may function as a positive regulator of osteoclast activity, proliferation, and differentiation [16,21]. Upon targeted overexpression of wildtype PTP-oc, osteoclast-like cells derived from human monocyctic U937 cells created resorption pits that were larger and deeper than those of
osteoclast-like cells derived from cells overexpressing phosphatase-deficient PTP-oc [16]. More importantly, more significant trabecular bone loss occurred because of increased bone resorption, but not decreased bone formation, in the osteoclastic lineage overexpressing PTP-oc in vivo [15,16,20]. PTP-oc stimulates osteoclasts in part by activating c-Src through dephosphorylation of its Tyr-527 (pY527) residue [15,16,22–24]. The suppression of PTP-oc expression by a PTP-oc antisense oligodeoxynucleotide in osteoclasts reduced their bone resorption activity and increased their c-Src pY527 level [15,24]. Collectively, these results suggest that PTP-oc dephosphorylates and activates c-Src in osteoclasts; therefore, the downstream effects of this signaling may be mediated via activation of NFêB and JNK2 [22]. In addition to its regulation of osteoclast activation of NFêB and JNK2 [22], the suppression of PTP-oc expression by a PTP-oc antisense oligodeoxynucleotide in osteoclasts reduced their bone resorption activity and increased their c-Src pY527 level [15,24].

To further study the molecular mechanism of PTP-oc signal transduction, we established an expression and purification system that is capable of providing a large-scale quantity of the functional intracellular catalytic domain of PTP-oc (ΔPTP-oc). We cloned the cDNA of ΔPTP-oc into the pET-28a (+) vector, purified the His6-tagged recombinant proteins using nickel affinity chromatography, and performed detailed characterization.

Materials and methods

Construction of expression vector

The pET-28a (+) plasmid was used to clone the ΔPTP-oc gene fragment. The cDNA fragment encoding the ΔPTP-oc (nucleotides 870–1656) was amplified by polymerase chain reaction (95 °C for 3 min; 94 °C for 1 min; 54 °C for 1 min; 72 °C for 1 min; 72 °C for 10 min; 30 cycles) from a human cDNA library (NCBI gene ID: NM_030669.2) and was ligated into the pET-28a (+) vector by T4-DNA ligase (New England Biolabs). The primers were designed to create Ncol and Xhol restriction sites to facilitate the insertion of the ΔPTP-oc fragment into the pET-28a (+) vector. The forward primer was 5’-AGCGGCGATCTGGAATTTTCTCTTCAGTTTGAGG-3’ and the reverse primer was 5’-GCATCTTCTGCCACACTGATG GATAAAAATG-3’. The ligated samples contained a C-terminal nucleotide sequence coding for six histidines, which was placed upstream and in frame with the D-terminus of the ΔPTP-oc fragment into the pET-28a (+) vector. The forward primer was 5’-AGCGGCGATCTGGAATTTTCTCTTCAGTTTGAGG-3’ and the reverse primer was 5’-GCATCTTCTGCCACACTGATG GATAAAAATG-3’. The ligated samples contained a C-terminal nucleotide sequence coding for six histidines, which was placed downstream and in frame with the ΔPTP-oc insert. The sizes of the polymerase chain reaction (PCR) products were confirmed by electrophoresis in a 2% agarose gel. A single colony obtained on Luria–Bertani (LB)-kanamycin plates was selected and cultured. The plasmids were purified and the sequence was confirmed by DNA sequencing. The recombinant plasmids were transformed into BL21 (DE3) cells for protein expression.

Expression of His6-tagged ΔPTP-oc

BL21 (DE3) cells that harbored the plasmid containing the ΔPTP-oc insert were grown in 2 ml of LB medium supplemented with 50 μg/ml kanamycin. After overnight growth at 37 °C with shaking, 2 ml of this pre-culture was used to inoculate 200 ml of fresh LB media containing an appropriate antibiotic in a flask, and the cells were grown at 37 °C to mid-log phase. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM to induce ΔPTP-oc expression, and the temperature was decreased to 16 °C to improve the soluble expression level [30]. After 24 h of growth at 16 °C, the cells were harvested at 4 °C by centrifugation at 5000g. The pellets were resuspended in 10 ml/g extraction buffer (50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM imidazole, 10% glycerol, 20 mM 2-mercaptoethanol, 2% Tween-20) containing a protease inhibitor mixture (1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin A, 2 μg/ml leupeptin and 1 μg/ml aprotinin) and broken up by sonication on ice. The lysate was cleared by centrifugation at 13,000g at 4 °C for 30 min and both supernatant and pellets were collected. The expression levels and solubility of ΔPTP-oc were assessed by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of ΔPTP-oc

The cytosolic supernatant from the above-mentioned step was loaded onto a Ni-NTA agarose column equilibrated with buffer A (50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 10 mM imidazole, 10% glycerol). The column was washed with buffer B (50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 10 mM imidazole, 10% glycerol). The tightly bound proteins were eluted with a linear imidazole gradient of 0–0.2 mol/L, and the protein peak was collected in fractions. The purity of soluble His6ΔPTP-oc was observed on 12% SDS gels. All purification procedures were carried out at 4 °C and PTP activity was analyzed by use of para-nitrophenylphosphate (p-NPP) as a substrate.

Protein concentration measurements and SDS–PAGE

The protein concentrations were determined using the Bradford method with bovine serum albumin (Sigma) as a reference standard according to the manufacturer’s instructions [31]. Soluble His6ΔPTP-oc was concentrated to 1 mg/ml and separated on 12% Tris–glycine SDS gels. Electrophoresis was performed in a Gibco-BRL Vertical Cell Electrophoresis Apparatus (Life Technologies) with standard molecular mass markers (Transgen Biotech). The gels were stained with Coomassie Blue for 1 h at room temperature. The destaining was performed in 10% acetic acid.

Western blot assays

The proteins were separated by 12% SDS–PAGE and then transferred to polyvinylidene difluoride (PVDF) membranes. Western blot analysis was performed by use of an anti-His6 tag monoclonal antibody (Abcam) as primary antibody. Proteins were detected using the enhanced chemiluminescence (ECL) method.

Enzymatic characterization of ΔPTP-oc

The phosphatase activity of the His6ΔPTP-oc fusion protein was tested in vitro by p-NPP (Sigma) hydrolysis assays at neutral pH, as previously described [13,27–29]. The reaction mixture consisted of 25 mM MOPS-NaOH (pH 7.0), 1 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml BSA, 0.05 μg/μl purified proteins and p-NPP at the indicated concentrations in a final volume of 100 μl. All activity assays were tested in triplicate. The blanks were prepared in the same manner as the samples but without the enzyme. The reaction was carried out at 37 °C for 15 min and terminated by the addition of 100 μl 0.2 M NaOH solution, and the assay results were read against a blank at 405 nm. One unit of activity (U) was defined as 1 nmol of phosphate released per min, and the specific activity was expressed as U/mg [32].

Kinetics of ΔPTP-oc

To study the enzyme kinetics of ΔPTP-oc, we performed enzyme assays with various concentrations of p-NPP substrate (S) for 15 min at 37 °C and pH 7.0. The data were processed with Graphpad Prism 4.0.
Results and discussion

Construction of an expression plasmid encoding ΔPTP-oc with a His tag

Primary structure analyses suggest that PTP-oc is a transmembrane protein [13,14,16]. The expression of the full-length enzyme resulted in the distribution of most of the protein in inclusion bodies with relatively little PTP activity in Escherichia coli [32]. This result made the purification of active PTP-oc from E. coli cells unfeasible.

The strategy to clone and express recombinant ΔPTP-oc involved inserting the gene of interest into the pET-28a (+) expression vector. Specifically, the insert encoding the phosphatase domain of PTP-oc, which was successfully amplified from the full-length human PTP-oc cDNA from pMD18-T, with the Ncol and Xhol restriction sites, was cloned into the pET-28a (+) vector containing a C-terminal His₆ tag to generate pET28a-ΔPTP-oc (Fig. 1A). Further characterization using specific restriction endonuclease digests and DNA sequencing demonstrated that the amplified target gene was inserted into the correct open reading frame of the vector (Fig. 1B). The resultant expression construct produced a fusion protein comprising ΔPTP-oc and a LEHHHHHH tag at the C-terminus with a predicted molecular weight of 31.2 kDa, for protein purification by metal affinity chromatography. The ligation products were then transformed into BL21(DE3) competent cells.

Expression of ΔPTP-oc in E. coli

The relationship between the temperature dependence of protein induction and inclusion body formation has been extensively studied in a variety of model systems [33,34]. Indeed, an apparent correlation has been noted. Whereby a decrease in the induction temperature gave rise to a lower propensity for recombinant protein expression in inclusion bodies and increased the solubility of recombinant PTPs [33,35,36]. Different temperatures were attempted after induction: 37°C, 32°C, 26°C, 20°C and 16°C. The temperature was finally lowered to 16°C post-induction which increased the soluble expression level. After determining the optimal conditions for ΔPTP-oc expression, the induction condition was optimized at 16°C with 0.1 mM of IPTG. These induction conditions were different from those used for purifying the catalytic region of rabbit PTP-oc [13], and from those used for purifying most PTPs [32,37,38]. E. coli cells were harvested and broken up after 24 h of induction, and total cell lysates were analyzed by SDS–PAGE (Fig. 2). A band at the expected molecular mass was detected and reached a high level of expression 24 h after induction. As Fig. 2 shows, this band was absent in the non-induced BL21 (DE3) lysate. Because the recombinant protein was highly overexpressed under these conditions, we established an induction time of 24 h for the large-scale production of ΔPTP-oc.

Purification of His₆-tagged ΔPTP-oc

His₆-ΔPTP-oc in crude extract was successfully bound to and competitively eluted from a metal-chelating affinity column after inclusion of a high concentration of imidazole (0.2 M). In the equilibrium buffer, a low concentration of imidazole (10 mM) reduced the nonspecific binding of non-tagged proteins, and 500 mM NaCl prevented undesirable ionic interactions. The purification yielded a significant amount of protein, with considerable enrichment of ΔPTP-oc. Peak fractions of proteins from the chromatographic sep-

![Fig. 1. Cloning of pET28a-ΔPTP-oc.](image1)

![Fig. 2. Recombinant ΔPTP-oc protein expression by SDS–PAGE.](image2)

![Fig. 3. Western blot analysis of recombinant ΔPTP-oc.](image3)
aration were resolved by SDS–PAGE and showed approximately 80% purity (Fig. 2). The identity of ΔPTP-oc was also confirmed by western blot analysis with an anti-His6 tag monoclonal antibody (Fig. 3). The molecular sizes of the recombinant proteins on SDS gels matched those predicted from the primary structures [13]. The efficiencies of the affinity purifications are shown in Table 1. The total protein yield was approximately 7 mg per 400 ml of cell culture.

Phosphatase activity of ΔPTP-oc

Because previous studies showed that PTPs are significantly active against p-NPP in vitro, the enzyme activity of ΔPTP-oc was assessed by measuring the ability of the protein to hydrolyze p-NPP [32,37,38]. The purified ΔPTP-oc was subjected to the PTP assay and showed highly efficient phosphatase activity for dephosphorylating p-NPP in a dose-dependent manner (Fig. 4). ΔPTP-oc also showed a time-dependent increase in the release of p-nitrophenol until the end of the reaction (Fig. 4). Based on these results, the mean ΔPTP-oc specific activity was calculated to be $1.57 \times 10^4$ U/mg, and was similar to those reported PTPs [37–40], suggesting that ΔPTP-oc maintains its native structure and remains intact after purification (Table 1).

Characterization of ΔPTP-oc

In general, most characterized PTPs and dual-specificity protein phosphatases display an optimal pH of approximately 5.0 when using low-molecular weight compounds as substrates [32,38,39]. However, ΔPTP-oc displayed an optimum pH of 7.0 when using p-NPP as a substrate (Fig. 5A). We also discovered a major effect on the activity at different temperatures (Fig. 5B). When the temperature was approximately 34°C, ΔPTP-oc showed maximum activity. At 37°C, the activity was 91.42% of that obtained at the

### Table 1

<table>
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<th>Fractions</th>
<th>Total protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Total activity (U)</th>
<th>Overall yield (%)</th>
<th>Purification (fold)</th>
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<tr>
<td>Cytosolic extract</td>
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<td>$4.20 \times 10^5$</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>NIA-agarose</td>
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<td>$1.57 \times 10^4$</td>
<td>$1.10 \times 10^7$</td>
<td>26</td>
<td>6.8</td>
</tr>
</tbody>
</table>

* PTP activity was analyzed using 10 mM p-NPP at pH 7.0. The protein concentrations were determined using the Bradford method, with BSA as a reference standard. The volume of cell culture was 400 ml.

Fig. 4. PTP assay of His6-ΔPTP-oc. Purified recombinant His6-ΔPTP-oc was assayed with two enzyme concentrations (0.1 μg/μl and 0.05 μg/μl) and different incubation times (5–30 min) in triplicate by use of p-NPP as a substrate. The relative activity is expressed as $A_{405}$. Samples without enzymes were used as negative controls.

Fig. 5. Effects of pH, temperature and ionic strength on the activity of ΔPTP-oc. The activity of ΔPTP-oc was analyzed with 10 mM p-NPP at different pH values and temperatures and in the absence of NaCl. (A) The buffers used were 25 mM sodium acetate (pH 3.5–5.5), 25 mM MOPS-NaOH (pH 5.5–7.5), and 25 mM Na$_2$B$_4$O$_7$·10H$_2$O·HCl (pH 8.0–9.0). The reaction temperature was 37°C. (B) In the absence of NaCl, 25 mM MES-NaOH (pH 7.0) was used in the p-NPP hydrolysis assay to test the effects of different temperature. (C) The effect of ionic strength on enzyme activity was tested in the presence of the indicated concentrations of NaCl at pH 7.0. The data represent the relative activities.

Fig. 6. Enzymatic kinetics of ΔPTP-oc. PTP activity assays were performed with different concentrations of p-NPP at pH 7.0, and the reaction velocity was calculated. (Reaction velocity = $A_{405}/(1.78 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \times \text{reaction time})$).
optimal temperature. It was observed that changes in ionic strength had a conspicuous effect on the activity on ΔPTP-oc (Fig. 5C).

Kinetics of ΔPTP-oc

We characterized the kinetics of ΔPTP-oc using p-NPP at pH 7.0. The data were analyzed with Graphpad Prism 4.0. The value of $V_{\text{max}}$ and $K_{\text{m}}$ of the enzyme with p-NPP as the substrate were estimated to be 6.1 µM/min and 801.8 µM, respectively (Fig. 6). In this case, ΔPTP-oc displayed low $K_{\text{m}}$ and high calculated $V_{\text{max}}$, which indicates that ΔPTP-oc is a high efficient enzyme.

Conclusions

Protein tyrosine phosphatases comprise a diverse super-family of enzymes that have fundamental roles in many biological processes including cell division, differentiation and gene expression [1,3,26]. PTPs are widely expressed in various cells, but their functions remain largely unknown [1,26,41]. It has been reported that rabbit osteoclastic PTP is expressed in the precursors of osteoclasts and has a positive effect on their functions [13,15,16,21,22,24,25]. In the present study, we achieved the soluble recombinant expression, purification and characterization of the intracellular phosphatase domain of ΔPTP-oc. We found that responses of ΔPTP-oc to temperature and ionic strength were similar to those of other PTPs. The optimal pH value of ΔPTP-oc is approximately 7.0, unlike other PTPs, whose optimal pH values are approximately 5.0. It is indicated that PTP-oc may play a different role in biological processes. This study provides a set of expression constructs that could serve in further structural and functional studies.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jpep.2014.11.008.

References


