Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase β (HPTPβ) using synthetic phosphopeptides

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The intracellular domain of human protein tyrosine phosphatase β (HPTPβ) (44 kDa) was expressed in bacteria, purified using epitope 'tagging' immunoaffinity chromatography, and characterized with respect to kinetic profile, substrate specificity and potential modulators of enzyme activity. A chromogenic assay based on the Malachite Green method was employed for the detection of inorganic phosphate (P_i) released from phosphopeptides by HPTPβ. This assay, modified so as to improve its sensitivity, was adapted to a 96-well microtitre plate format, and provided linear detection between 50 and 1000 pmol of P_i. The cytoplasmic domain of HPTPβ was strongly inhibited by vanadate, molybdate, heparin, poly(Glu, Tyr) (4:1) and zinc ions. In order to explore the substrate preferences of this PTPase, we generated 13-residue synthetic phosphotyrosine-containing peptides that corresponded to sites of physiological tyrosine phosphorylation. HPTPβ demonstrated $k_{cat.}$ values between 76 and 258 s^{-1} using four different phosphopeptides. The substrate preference of HPTPβ was in the order $src^{Tyr-527}$ > PDGF-$R^{Tyr-740}$ > ERK$1^{Tyr-204}$ ≫ CSF-$1R^{Tyr-708}$ with K_m values ranging from 140 μM to greater than 10 mM. The variations in affinity were probably due to differences among the four phosphopeptides compared, particularly with respect to the character of the charged amino acids flanking the phosphotyrosine residue.

INTRODUCTION

The protein tyrosine phosphatases (PTPases) comprise a growing family of molecules demonstrating a complexity of structure that appears to exceed that of the protein tyrosine kinases (PTKs) (Hunter, 1989; Charbonneau and Tonks, 1992; Pot and Dixon, 1992). Both groups of molecules are important elements in signal transduction pathways within eukaryotic cells (Ullrich and Schlessinger, 1990; Cantley et al., 1991). The diversity of structure and the differential patterns of tissue-specific expression suggest the possibility of multiple interactions taking place between these two groups of molecules during cell proliferation, differentiation and metabolism. In reversing tyrosine phosphorylation, PTPases potentially regulate such important processes as: the ligand-induced autophosphorylation of PTK growth factor receptors (Ullrich and Schlessinger, 1990), the phosphotyrosine-mediated binding of *src* homology 2 (SH2)-domain-containing proteins to autophosphorylated PTK growth factor receptors (Koch et al., 1991), and the activation state of the *src* family of PTKs (Hunter, 1987; Cantley et al., 1991).

While the PTPases show considerable heterogeneity within their non-catalytic domains (Charbonneau and Tonks, 1992; Pot and Dixon, 1992) their catalytic domains show striking evolutionary conservation. PTPases isolated from prokaryotic organisms (Guan et al., 1991a), from yeast (Ottilie et al., 1991; Guan et al., 1991b; Ota and Varshavsky, 1992), *Styela plicata* (Matthews et al., 1991), *Drosophila melanogaster* (Streuli et al., 1989; Hariharan et al., 1991; Tian et al., 1991; Yang et al., 1991), *Caenorhabditis elegans* (Matthews et al., 1991) and mammals (Fischer et al., 1991) all share characteristic stretches of amino acids including the [I/V]HCXAGXXR[S/T]G motif that defines the family. Both intracellular and receptor-like PTPases have been identified. Most receptor-like PTPases identified, such as the leucocyte common antigen (LCA or CD45), leucocyte common antigen-related phosphatase (LRP; PTPα) and the leucocyte-common-antigen-related protein (LAR), for example, possess two tandemly arranged catalytic domains (Charbonneau and Tonks, 1992; Pot and Dixon, 1992). Although the function of the second catalytic domain of these PTPases is unclear, it may serve to regulate the specificity or activity of the first PTPase domain (Streuli et al., 1990; Wang and Pallen, 1991). Alternatively, these domains may become catalytically active under as yet undefined conditions.

Human PTPβ (HPTPβ) was selected for initial characterization as part of a comparative study to examine whether different classes of PTPases were capable of showing substrate-specific differences in activity. Although belonging to the group of receptor-like PTPases, both HPTPβ (Krueger et al., 1990) and its probable homologue, isolated from *Drosophila*, DPTP10D appear to be unique in that they possess only a single catalytic domain. DPTP10D is expressed in the central nervous system of the developing *Drosophila* embryo, where it has a potential role in axonal outgrowth and guidance (Tian et al., 1991; Yang et al., 1991). The HPTPβ cDNA is predicted to encode a protein having domains of 1599, 21 and 355 amino acid residues, corresponding to the extracellular, transmembrane and intracellular segments respectively (Krueger et al., 1990). The HPTPβ gene has been localized to human chromosome 12q15–q21 (Harder et al., 1992). While the extracellular region of HPTPβ contains a tandem array of 17 approx. 19-amino-acid-residue segments each containing a fibronectin type-III repeat, the extracellular domain of DPTP10D consists of 12 such units (Tian

Abbreviations used: CSF-1R, colony stimulating factor-1 receptor; ERK, extracellular signal-regulated kinase; HPTPβ, human protein tyrosine phosphatase β; IPTG, isopropylthiogalactoside; LAR, leucocyte-common-antigen-related protein; PAO, phenylarsine oxide; PDGF-R, platelet-derived growth factor receptor; *p*-NPP, *p*-nitrophenol phosphate; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; SH2, src homology-2 domain; TFMSA, trifluoromethanesulphonic acid; LRP, leucocyte common antigen-related phosphatase; *p*-NPP, *p*-nitrophenol phosphate; DTT, dithiothreitol.

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et al., 1991). Similar repeats have been found in membrane proteins involved in cell–cell interactions, such as the neural cell adhesion molecule N-CAM (Edelman, 1987).

Although individual PTPases will probably be found to demonstrate selectivity towards particular phosphoprotein substrates *in vivo*, the potential role of the intracellular domain in such selectivity is unknown. One approach to this problem is to first demonstrate whether intracellular PTPase domains are able to show substrate specificity *in vitro*. For such a study, the intracellular domain of HPTPβ was selected for analysis. The single catalytic domain structure of this PTPase makes it ideal for addressing questions regarding the specificity and kinetics of the intact cytoplasmic domain of a receptor-like PTPase. The use of HPTPβ avoided potential complications inherent to an analysis of double-catalytic-domain PTPases due either to the presence of the second PTPase domain, or to mutations that have been introduced to remove the second domain. For a kinetic analysis of HPTPβ a set of synthetic phosphopeptide substrates based on sites of tyrosine phosphorylation *in vivo* was employed.

EXPERIMENTAL

General materials

A human placental λ Zap cDNA library was obtained from Stratagene (catalogue no. 936203). Vent DNA polymerase was from New England Biolabs. Other enzymes and protease inhibitors were from Boehringer–Mannheim. The pET11a bacterial expression vector and the bacterial strain *Escherichia coli* BL21 (DE3) were from Novagen (Studier et al., 1990). Mouse ascites fluid containing the monoclonal antibody 12CA5 was from BAbCO (Field et al., 1988). The DNA-sequencing kit, MonoQ column, protein A and cyanogen bromide–Sepharose were obtained from Pharmacia LKB Biotechnology. A Biotec model 309 microtitre plate reader was used. Half-volume microtitre plates were from Costar. Malachite Green [1 % (w/v) solution] was from BDH. Ammonium molybdate and *p*-nitrophenol phosphate (*p*-NPP) were from Sigma and Tween 20 was from Bio-Rad. *t*-Butyloxycarbonyl (t-Boc)-dibenzyl-tyrosine phosphate was obtained from Peninsula Laboratories. All other reagents and chemicals were from BDH or Sigma.

Preparation of Malachite Green reagent

To prepare the Malachite Green solution, one volume of 4.2 % (w/v) ammonium molybdate in 4 M HCl was added to 3 vol. of 0.045 % (w/v) Malachite Green. This solution was stirred for a minimum of 30 min before syringe filtering through a 0.22 μm-pore-diam. filter (Millipore). The filtered solution was stored for up to 6 months at 4 °C. Tween 20 (0.01 %, v/v) was added to an aliquot of filtered Malachite Green solution before use.

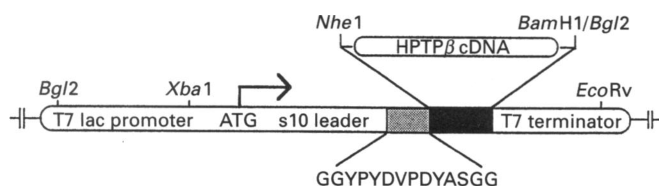

Figure 1 Construction of the modified pET11a expression vector

Nucleotides encoding the 'tag' peptide sequence GGYPYDVPDYASGG were introduced into the plasmid pET11a (Studier et al., 1990) upstream of the HPTPβ cDNA fragment as described in the text. The 'ATG' and arrow indicate the translational start codon of the fusion protein.

Composition of buffers

Buffer A: 25 mM Tris/HCl, pH 7.5/50 mM NaCl/5 mM 2-mercaptoethanol. Buffer B: same as buffer A except that soybean trypsin inhibitor (0.6 μg/ml), leupeptin (0.5 μg/ml), phenylmethanesulphonyl fluoride (1 mg/ml) and pepstatin (0.7 μg/ml) were added. Buffer C: 25 mM Tris/HCl, pH 7.5/250 mM NaCl/5 mM 2-mercaptoethanol/0.5 % (v/v) Tween-20. Buffer D: 25 mM Tris/HCl, pH 7.5/50 mM NaCl/5 mM 2-mercaptoethanol/1 mg/ml decapeptide (YPYDVPDYAS). Buffer E: 100 mM Tris/HCl, pH 8.0/5 mM 2-mercaptoethanol.

Cloning of the intracellular domain of HPTPβ

The sequences of the HPTPβ primers: the 5′ primer CTCTCGCTAGCAGACAGAAAGTGAGCCATGGTCGAG and the 3′ primer CTCAGATCTCTCAATGCCTTGAATAG-ACTGGATC were derived from the published sequence of HPTPβ (Krueger et al., 1990), and synthesized on an Applied Biosystems DNA synthesizer (model 391). The 5′ and 3′ primers correspond to nucleotides 1594–1619 and 1900–1926 respectively of HPTPβ. The 5′ primer contains additional nucleotides for an *Nhe*1 restriction endonuclease site, while the 3′ oligonucleotide contained a *Bgl*II site. A sample (15 μl) of an amplified human placental λ Zap cDNA library was combined with an equal volume of ^2H_2O, heated at 70 °C for 10 min and then chilled on ice. To this reaction mixture 200 μM dNTPs, 5 μl of 10 × Vent buffer, 5 μl of 10 × BSA (10 mg/ml), and 3 units of Vent DNA polymerase were added to a final volume of 50 μl. PCR amplification (30 cycles of 94 °C for 1.5 min, 50 °C for 1.75 min, and 72 °C extension for 2 min) produced a 1.07 kb fragment. This fragment was gel-purified and ligated into the *Eco*Rv site of Bluescript (Stratgene) to give pHPTPβ. The insert was verified by DNA sequencing (Sanger et al., 1977). pHPTPβ was then digested with *Nhe*1 and *Bgl*II and the HPTPβ cDNA fragment was subcloned into *Nhe*1- and *Bam*H1-digested pET11a plasmid, yielding pET-HPTPβ.

Modification of pET11a bacterial expression vector

To facilitate detection and purification of recombinant proteins expressed in bacteria the pET11a vector was modified by the addition of a nucleic acid sequence encoding a 10-amino-acid-residue segment located at the N-terminus of the expressed protein. This 10-residue sequence corresponds to a peptide derived from the influenza virus haemagglutinin protein that is recognized by the monoclonal antibody 12CA5 (Field et al., 1988). PCR was used to incorporate this decapeptide 'tag' sequence, along with two flanking glycine spacers, into the pET11a plasmid (Figure 1). Samples (50 pmol) of each of primer-A (GGATCGAGATCTCGATCCCGCGAAATT) and primer-B (CTCGCTAGCACCGCCAGATGCGTAGTCCGGAACG-TCGTACGGGTAACCGCCGCGGCCCATTTGCTGTC-CACCAGTCATGCTAGTCATATG) containing the fusion sequence were added to 100 ng of pET11a template DNA, 10 × PCR buffer, 200 μM dNTPs and 2.5 units of *Taq* DNA polymerase in a volume of 100 μl. The fragment resulting from PCR amplification (30 cycles of 96 °C for 45 s, 50 °C for 90 s, 72 °C for 120 s) was subcloned into Bluescript plasmid, the DNA sequence determined and then directionally subcloned into

*Bgl*II- and *Nhe*1-digested pET11a to give pET-HPTPβtag for bacterial expression.

Bacterial expression of HPTPβ

The pET-HPTPβtag plasmid was transformed into competent BL21 (DE3) bacteria (Studier et al., 1990). A 20 ml overnight culture of pET-HPTPβtag in BL21 (DE3) bacteria grown in 100 μg/ml ampicillin/Luria broth was added to 1 litre of this same media and grown with shaking to an absorbance of 0.7 at 37 °C in the absence of isopropylthiogalactoside (IPTG). Recombinant protein synthesis was stimulated by the addition of 1 mM IPTG. After 4–5 h of induced growth the bacterial cells were pelleted by centrifugation at 3500 rev./min (3000 *g*) for 15 min at 4 °C. The cells were washed in buffer A, sedimented and then resuspended in 5 ml of buffer B. Lysozyme (10 mg/ml; 550 μl) was added and the mixture was incubated on ice for 15 min. Three cycles of alternating freeze–thaws on solid CO_2/ethanol were performed followed by addition of 55 μl of 1 M $MgCl_2$ and 25 μl of DNAase 1 (10 mg/ml) to the lysate. After incubation at 250 °C for 20 min, 100 μl of 500 mM EDTA and 600 μl of 10 % (v/v) Triton X-100 were then added and the lysate was then held for 30 min at 25 °C before ultracentrifugation (Sorvall RC 80, SW55Ti rotor) at 35000 rev./min. (150000 *g*) at 4 °C for 45 min. The supernatant was kept.

PTPase-'Tag' purification by affinity chromatography

Monoclonal antibody 12CA5 was purified from ascites fluid by passage over a sheep anti-(mouse IgG) cyanogen bromide-linked Sepharose column. A 12CA5 affinity column was prepared by cross-linking 4 mg of the antibody (IgG2b isotype) to 2 ml of Protein A–Sepharose using dimethylpimelimidate (Harlow and Lane, 1988). The supernatant from the bacterial extract was then added to a Protein A–12CA5 affinity column and rotated at 4 °C overnight. The protein–bead matrix was washed with 50 ml of buffer C at 4 °C. Elution of HPTPβ bound to the column was achieved by flooding the column with 1.5 ml of buffer D warmed to 30 °C containing 1 mg/ml of the decapeptide (YPYDVPDYAS) (Field et al., 1988). The column mixture was incubated for 15 min at 30 °C before collection of the eluate. This elution procedure was repeated and the two elutions were combined and concentrated to a volume of approx. 500 μl with a Centricon 30 microconcentrator (Amicon). The concentrated eluate was then loaded on to a MonoQ anion-exchange column (1 ml bed volume) equilibrated in 25 mM Tris/HCl, pH 7.5/5 mM DTT. The column was eluted with a 0–1.0 M NaCl gradient using a Pharmacia f.p.l.c. system. PTPase activity in 250 μl column fractions was assessed as described below. Peak fractions were pooled, glycerol was added to a final concentration of 20 % and then aliquots were frozen in liquid nitrogen before storage at -80 °C. The 12CA5 affinity column was regenerated for repeated use by eluting the decapeptide with 10 column vol. of 100 mM triethylamine, pH 11.5, followed by equilibration in 50 mM Tris/HCl, pH 7.5/150 mM NaCl.

Synthesis and analysis of phosphotyrosine-containing peptides

Peptides were synthesized using solid-phase methods with t-Boc α-protected amino acids, with appropriate side-chain protection, as described (Clark-Lewis et al., 1991). Phosphopeptides were synthesized with *t*-Boc-dibenzyl-tyrosine phosphate. After chain assembly, the peptides were de-protected using trifluoromethane sulphonic acid (TFMSA). Each 100 mg of peptide resin was stripped with 100 μl of thioanisole, 50 μl of ethanedithiol and 1 ml of trifluoroacetic acid. TFMSA (100 μl) was added and allowed to react for 2 h at room temperature. The peptide was precipitated with diethyl ether, washed and then dissolved in 6 M guanidine hydrochloride, pH 8.5. For purification this mixture was loaded directly on to a reverse-phase h.p.l.c. column (Clark-Lewis et al., 1991). The mass of each peptide was confirmed using ion-spray m.s. on a model API III triple-quadrapole mass spectrometer (SCIEX, Thornhill, Ont., Canada) with a liquid-delivery interface. The amino acid composition was confirmed by amino acid analysis. Phosphopeptides were carefully dried before weighing on an analytical balance and dissolved in the appropriate volume of ^2H_2O. Peptides with lower solubility were dissolved in ^2H_2O to saturation and centrifuged at 14000 rev./min. (15000 *g*) and the supernatants saved. The concentration of each phosphopeptide solution was determined by measuring P_i release by acid hydrolysis (Hasegawa et al., 1982) using the Malachite Green microtitre-plate assay. Briefly, the phosphopeptides were completely hydrolysed by acid cleavage in 50 % (v/v) perchloric acid and 50 % (v/v) sulphuric acid at 150 °C for 16 h. Liberated P_i was then detected using the Malachite Green assay. Values for P_i release were then obtained by comparison with the Malachite Green standard curve. This allowed equimolar concentrations of each peptide to be determined and was particularly useful with peptides that demonstrated incomplete solubility.

Enzyme kinetics

Enzymic activity of HPTPβ on various phosphotyrosine-containing substrates was determined using a Malachite Green microtitre-plate assay. Enzyme reactions were carried out on half-volume 96-well plates in a final volume of 25 μl. Appropriately diluted enzyme, substrate peptides and potential modifiers of enzyme activity, were incubated for 5–15 min at 25 °C in buffer E at pH 8.0. Reactions were terminated by the addition of 50 μl of Malachite Green solution and the plates were then incubated for 15 min at room temperature before measurement of absorbance at 620 nm. The detection of MonoQ Sepharose column fractions containing HPTPβ was achieved by assaying for PTPase activity essentially as described above, except that 5 mM *p*-NPP was used as substrate. Reactions were carried out for 15 min before addition of 50 μl of Malachite Green solution and absorbance determination.

RESULTS AND DISCUSSION

Expression of HPTPβ in bacteria

To express HPTPβ in bacteria, the cDNA sequence encoding the entire cytoplasmic domain of HPTPβ was cloned from a human placental cDNA library using Vent DNA polymerase. This polymerase was selected for the PCR amplification in view of its reported proof-reading ability (New England Biolabs). The cDNA obtained was then introduced into the pET11 bacterial expression vector (Studier et al., 1990). This vector was modified so it contained an epitope 'tag' decapeptide sequence derived from the influenza haemagglutinin protein (Field et al., 1988). The HPTPβ cDNA was cloned into pET11tag and expressed as a fusion protein that consisted of the 12-residue N-terminus S-10 leader peptide of the pET11a vector, and the decapaptide 'tag' flanked by two glycine 'spacer' residues (Figure 1). The epitope 'tag' provides a means for immunoaffinity purification as well as

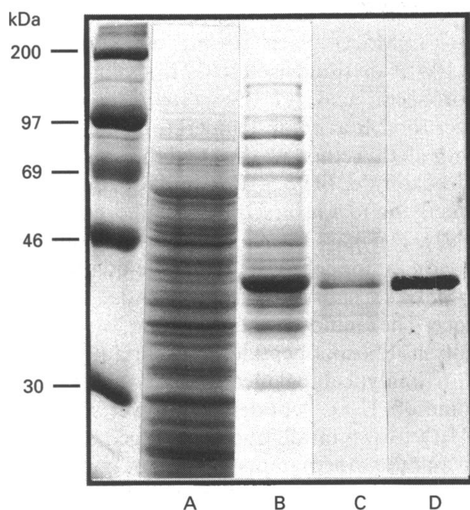

Figure 2 SDS/PAGE analysis of HPTPβ purification

Proteins were separated on a 12% (w/v) polyacrylamide gel and stained with Coomassie Blue (lanes A, B and C), or transferred to nitrocellulose for immunoblotting with the 12CA5 monoclonal antibody (lane D) in conjunction with alkaline phosphatase-linked goat anti-(mouse IgG heavy and light chain-specific) antisera (Calbiochem). Lane A, raw bacterial lysate; lane B, protein eluate from the 12CA5 immunoaffinity column; lane C, HPTPβ following MonoQ chromatography; and lane D, immunoblot of the purified HPTPβ intracellular domain.

immunodetection of proteins expressed in bacteria using this vector (Figure 2).

HPTPβ was purified (Figure 2) for kinetic studies by immunoaffinity purification on a 12CA5 column and MonoQ Sepharose chromatography, as outlined in the Experimental section. Typically, between 10 and 50 μg of HPTPβ were recovered from the 12CA5 column. Increased recovery of the protein was achieved by re-application of the bacterial lysate to regenerated affinity columns. As evident from lane A (Figure 2), even in IPTG-induced cultures HPTPβ protein constituted only a minor band within the crude extract. Immunoblotting experiments using the monoclonal antibody 12CA5, however, revealed excellent IPTG induction of HPTPβ protein expression from the pET plasmid (results not shown). In subsequent experiments employing the pGEX bacterial expression system (Smith and Johnson, 1988) considerably higher yields of protein were obtained, with HPTPβ being the major band even within the crude bacterial extracts (results not shown). However, despite the relatively low expression level of HPTPβ in the pET system, sufficient quantities of enzyme were obtained to enable the characterization of this enzyme. Purified HPTPβ was quantified by comparison with serially diluted BSA and ovalbumin standards. The enzyme was stable for over 4 months when stored in 20% (v/v) glycerol at −80 °C, or for 3–4 weeks when stored at 4 °C. The enzyme, however, became inactive when subjected to repeated freeze–thawing.

IPTG-induced bacterial cell extracts, lacking the HPTPβ expression vector, were also subjected to 12CA5 monoclonal antibody immunochromatography. Using phosphopeptides or *p*-NPP as test substrates, no phosphatase activity was detected within bacterial proteins adhering non-specifically to the 12CA5 affinity column, thus P_i release from the substrates employed in this study was the result of HPTPβ activity alone.

Preparation of phosphotyrosine peptide substrate

To study both the reaction kinetics of HPTPβ and the substrate preferences of this PTPase, four 13-residue peptides were synthesized containing phosphotyrosine residues corresponding to sites of phosphorylation *in vivo*. As little information exists regarding the three-dimensional structure of tyrosine phosphorylation sites within intact proteins, the assumption was made that such sites might be located at the centre of an exposed segment of primary sequence. Thus, the phosphotyrosine residue was placed centrally within the synthetic peptides. Peptides having 13 residues were selected: (i) to maintain as much of the sequences normally surrounding the phosphotyrosine residues as possible, while yet being of a length that minimized the potential for peptide-specific secondary structures; and (ii) for the heterogeneity with respect to both their roles *in vivo* and to the composition of the residues flanking the phosphotyrosine sites.

The following phosphotyrosine-containing peptides were synthesized (Table 1). (1) TSTEPQYQPGENL, containing the C-terminal phosphorylation site of c-src. This sequence contains Tyr-527 which has been associated with the negative regulation of c-src kinase activity (reviewed in Hunter, 1987). (2) TGFLTEYVATRWY, corresponding to the sequence containing Tyr-204 of the human extracellular signal-regulated kinase (ERK) 1 kinase. Phosphorylation of ERK1 on Tyr-204 and Thr-202, is associated with increased activity of this serine/threonine kinase (Anderson et al., 1990; Payne et al., 1991). (3) GESDGGYMDMSKD, corresponding to the sequence containing Tyr-740 of the human platelet-derived growth factor receptor (PDGF-R) β-chain. Phosphorylation of the β-chain of the PDGF-R on Tyr-740 and Tyr-751 has been shown to mediate phosphatidylinositol 3′-kinase association with the activated receptor both *in vitro* and *in vivo* (Escobedo et al., 1991; Kashishian et al., 1992). (4) IHLEKKYVRRDSG, corresponding to the sequences flanking Tyr-708 of the human colony-stimulating factor-1 receptor (CSF-1R). Although the CSF-1R$^{Tyr-708}$, and its mouse counterpart Tyr-706, are known to be

Table 1 Kinetic parameters of HPTPβ

Kinetic data were generated under conditions in which less than 20% of substrate was converted into product, and were analysed using the enzyme kinetics program Enzfitter (Biosoft U.K.). All assays were performed in duplicate with similar results obtained in two or more separate experiments. Sequences are given in the one-letter code for amino acids. (Y*, phosphotyrosine).

Substrate	K_m (mM)	$V_{max.}$ (μmol/mg per min)	$K_{cat.}$ (s^{-1})
TSTEPQY*QPGENL (src$^{Tyr-527}$)	0.142±0.009	350±10	258
GESDGGY*MDMSKD (PDGF-R$^{Tyr-740}$)	0.175±0.007	272±4	200
TGFLTEY*VATRWY (ERK1$^{Tyr-204}$)	0.330±0.03	240±13	177
IHLEKKY*VRRDSG (CSF-1R$^{Tyr-708}$)	> 10.0	163	120
p-NPP	2.5±0.5	103±9	76

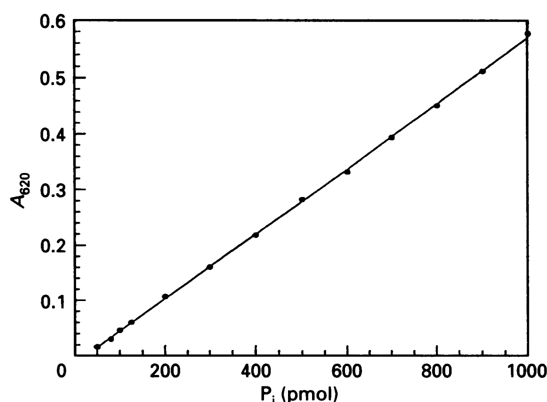

Figure 3 Standard curve of phosphate detection using the Malachite Green microtitre-plate assay

Inorganic phosphate standards were prepared from KH_2PO_4. All points represent duplicate assays from at least two independent experiments. Standards were prepared from KH_2PO_4 that had been desiccated at 80 °C for 5 h. Appropriately diluted P_i standards in a volume of 25 μl were delivered to half-volume wells of a 96-well microtitre plate, followed by the addition of 50 μl of the Malachite Green Tween 20 solution. After 15 min of colour development at room temperature the absorbance of each well was measured in an e.l.i.s.a. plate reader at 620 nm.

phosphorylated following stimulation with CSF-1 (Van der Geer and Hunter, 1990; Reedijk et al., 1992), the function of this phosphorylation is unknown. As the sequence surrounding this tyrosine is identical in the CSF-1R of both species, a conserved function is suggested for this region.

The product of each synthesis corresponded to a single major peak on h.p.l.c., with minor side products. After h.p.l.c. purification a single molecular species that corresponded to the expected mass of the phosphopeptide was observed in each case. We found the most reliable way to quantify the concentration of the phosphopeptide within the working solutions, was to hydrolyse the samples completely in a perchloric and sulphuric acid mixture at high temperature, as described in the Experimental section. P_i thus released from the phosphopeptides could then be accurately quantified with the Malachite Green assay via comparison with the standard curve generated for P_i detection shown in Figure 3.

Malachite Green microtitre-plate assay

The release of P_i from the various synthetic phosphopeptides was determined using a modification of the Malachite Green assay (Hess and Derr, 1975) that has been used in the analysis of calcineurin (Martin et al., 1985) and more recently to study the activities of the LAR and CD45 PTPases on synthetic phosphopeptides (Cho et al., 1991, 1992). The Malachite Green assay has provided linear detection between 1 nmol and 10 nmol of P_i (Lanzetta et al., 1979; Cho et al., 1991, 1992).

The sensitivity of this assay was improved: Figure 3 illustrates a P_i standard curve generated with the microtitre-plate assay, P_i detection is linear between 50 pmol and 1000 pmol. While 1 nmol of P_i yields an absorbance of 0.1 unit in the standard assay, the microtitre-plate assay reported here achieved an absorbance of 0.6 for the same quantity of P_i. The detergent Sterox, used in the standard assay, was replaced by Tween 20. Addition of Tween 20 (0.01 %, v/v) to the Malachite Green solution was required for linear P_i detection above 300 pmol (results not shown). With the microtitre-plate assay, enzyme reactions and released P_i detection were carried out in half-volume microtitre-plate wells, eliminating the need for sample transfer and chamber cleaning associated with the use of cuvettes when a spectrophotometer is used for P_i detection. The microtitre-plate reader also has the advantage of allowing absorbance determination of multiple reaction samples simultaneously.

PTPase activity modifiers

The activity of HPTPβ was tested in the presence of a variety of potential modifiers (Table 2). HPTPβ was inhibited by the cations Ca^{2+}, Mg^{2+} and Mn^{2+}. Mn^{2+} showed the strongest inhibitory effect of the three cations tested, with 26 % of control activity at 10 mM. The inhibition observed, however, required high concentrations of these three cations. Itoh et al. (1992) found that both Ca^{2+} and Mg^{2+} stimulated HPTPβ activity at concentrations of 10 mM. Interestingly, we found that when the substrate concentration was increased 10-fold, from 200 μM to 2 mM of $src^{Tyr-527}$, the inhibitory effects of these cations were reduced, and instead a stimulation of 107 % and 110 % was observed using 10 mM Ca^{2+} and Mg^{2+} respectively. Addition of the serine/threonine protein phosphatase inhibitor NaF led to a marginal stimulation of HPTPβ activity at a concentration of 10 μM, whereas 72 % of control activity was observed at 10 mM NaF. In contrast, Zn^{2+} was a potent inhibitor of HPTPβ activity in low micromolar concentrations.

The PTPase inhibitors vanadate and molybdate inhibited HPTPβ activity. The IC_{50} for vanadate was 0.45 μM, while that of molybdate was 60 μM. Phenylarsine oxide (PAO) has been reported to be a potent inhibitor of the PTPase CD45, with an IC_{50} between 5 μM and 10 μM (Garcia-Morales et al., 1990). In this study PAO stimulated activity of HPTPβ at concentrations reported to strongly inhibit CD45. PAO has been reported to have no effect on rat LAR at concentrations 10-fold higher than those used to inhibit CD45 (Pot et al., 1991). Together these results suggest that PAO may be specific for CD45, or perhaps for a subgroup of PTPases.

The polycation spermine has been reported to stimulate the catalytic activity of HPTPβ by 244 % and 265 % at 10 μM and 100 μM respectively, while causing inhibition at 10 mM when tested on the artificial substrate [^{32}P]Raytide (Itoh et al., 1992). The effects of various concentrations of spermine on the activity of HPTPβ on the substrates $src^{Tyr-527}$ (Table 2) and *p*NPP (results not shown) were tested. Spermine had little or no effect at 20 μM and 200 μM, whereas inhibition by spermine was observed on both substrates tested at concentrations of 2.0 mM and 20 mM. The nature of the differences between these results and those of Itoh et al. (1992) with respect to the effects of spermine, Ca^{2+} and Mg^{2+} on the activity of HPTPβ are unclear. They may reflect the nature of the substrates used, or the conditions used in the enzyme assays. Alternatively, differences in the fusion sequences at the N-terminus of the bacterially expressed proteins might have affected their susceptibility to the modulators.

The polyanion heparin and poly(Glu,Tyr) (4:1) both strongly inhibited the activity of HPTPβ at 1.0 μM. Inhibition was observed with both compounds at concentrations as low as 10 nM. Kinetic analysis of placental PTPases (Tonks et al., 1988) indicated that the poly(Gly,Tyr) (4:1) inhibition of PTPase 1B was non-competitive, and suggested a binding site for polyanionic molecules distinct from the active site. Studies of the effects of various polyanionic and polycationic amino acids polymers such as poly(Glu,Tyr) (4:1) on the enzymic activities of HPTPβ, LAR and CD45 have revealed a positive correlation between the

Table 2 Modulators of HPTPβ activity with 200 μM src$^{Tyr-527}$ peptide

All assays were performed in duplicate, in two or more separate experiments.

Effector	Concentration	PTPase activity (% of control)
NaF	1 mM	105
	10 mM	72
EDTA	1 mM	98
	10 mM	81
Spermine	2 mM	85
	20 mM	19
Heparin	1 μM	32
	10 μM	17
Poly(Gly/Tyr) (4:1)	0.01 μM	80
	1 μM	33
PAO	0.1 mM	110
	1 mM	104
Vanadate	1 μM	32
	10 μM	< 5
Ammonium molybdate	0.01 mM	76
	0.1 mM	38
	1 mM	5
$CaCl_2$	10 mM	72
	25 mM	34
	50 mM	15
$MgCl_2$	10 mM	66
	25 mM	37
	50 mM	11
$MnCl_2$	10 mM	23
	25 mM	10
	50 mM	< 5
$ZnCl_2$	1 μM	70
	25 μM	40
	75 μM	24

tyrosine content of such polymers and their potency as inhibitors (Itoh et al., 1992).

Kinetic analysis of HPTPβ using phosphotyrosine-containing peptides

The specificity of HPTPβ for phosphotyrosine was tested using the free amino acids phosphotyrosine, phosphothreonine and phosphoserine as substrates. While HPTPβ catalysed the hydrolysis of phosphotyrosine it showed no detectable activity towards the other phosphoamino acids tested (results not shown).

Using the first catalytic domain of LAR, the affinity of this PTPase for different synthetic phosphopeptides was found to vary between 27 μM (a 12-mer) and 4.1 mM (a 6-mer), a 150-fold difference (Cho et al., 1991). However, the peptides selected for this analysis of LAR were not uniform, varying between six and 12 residues in length. Variation, with a range of two to eight residues, was also present with respect to the number of residues flanking a given phosphotyrosine. Thus, some of the observed differences in the affinity of LAR for these substrates was probably due to the overall design of the synthetic peptides.

In this kinetic study of HPTPβ, the phosphopeptides selected were homogeneous with respect to length as well as to placement of the phosphotyrosine residue within the sequence (Table 1). The results of the kinetic analysis of HPTPβ are presented in Table 1. $k_{cat.}$ values ranged between 76 and 258 s^{-1}, based on a molecular mass for HPTPβ of 44 kDa. A $k_{cat.}$ of 258 s^{-1} for the phosphopeptide src$^{Tyr-527}$ is approx. 3.7 times that observed for LAR-D1 (Cho et al., 1991) on a similar src$^{Tyr-527}$ phosphopeptide. Comparison of the catalytic activity of HPTPβ on src$^{Tyr-527}$ with that previously reported (Cho et al., 1992) revealed that HPTPβ had a $V_{max.}$ of approx. twice that of CD45, and six times greater than that of LAR-D1. In addition, the $V_{max.}$ obtained with the src$^{Tyr-527}$ substrate is approx. 30-fold greater than that reported (Wang and Pallen, 1992) for HPTPβ using phosphorylated RR-src peptide as a substrate. Although this difference in activity may have been due to differences intrinsic to the peptide substrates employed, it probably resulted from differences in the pH of the enzyme assays: while Wang and Pallen (1992) assayed HPTPβ activity at pH 6.0, in the current study, this enzyme was found to favour more alkaline conditions, with a narrow optimum centred about pH 8.0 (results not shown). This was also in keeping with a similar pH optimum that has been reported for this PTPase (Cho et al., 1992).

A K_m value of 140 μM for the src$^{Tyr-527}$ phosphopeptide was similar to that of 170 μM obtained for the PDGF-R$^{Tyr-740}$ peptide. The K_m for the ERK1$^{Tyr-204}$ peptide at 330 μM was approx. twice that of either the src$^{Tyr-527}$ or the PDGF-R$^{Tyr-740}$ peptide. Peptide CSF-1R$^{Tyr-708}$ was a relatively poor substrate for HPTPβ. The K_m value for this peptide was greater than 10 mM, approx. 90-fold higher than that of the src$^{Tyr-527}$ peptide. The sequences surrounding the tyrosine residue of the CSF-1R peptide are unusual as compared with the majority of sites of tyrosine phosphorylation that have been identified (Pearson and Kemp, 1991), in that the phosphotyrosine residue is flanked on the N-terminal side by two basic (Lys-Lys) residues and by two additional basic (Arg-Arg) residues on the C-terminal side of the phosphotyrosine residue. Peptide ERK1$^{Tyr-204}$ contains an acidic (Glu) residue neighbouring Tyr-204. In contrast, peptides src$^{Tyr-527}$ and PDGF-R$^{Tyr-740}$, the best substrates of HPTPβ in this study, contained uncharged amino acids surrounding the phosphotyrosine residue. HPTPβ catalysed the removal of phosphate from p-NPP with a K_m of 2.5 mM, a K_m superior to that for peptide CSF-1R$^{Tyr-708}$. This finding suggests that the peptide sequence surrounding the phosphotyrosine residue of CSF-1R$^{Tyr-708}$ inhibits HPTPβ recognition of this site.

While the CSF-1R$^{Tyr-708}$ peptide is a relatively poor substrate for HPTPβ, this does not necessarily hold true for other PTPases. For example, the bacterially expressed first catalytic domain (D1) of LRP (PTPα), which might be considered analogous to the single catalytic domain of HPTPβ, yielded a K_m of 184 μM with the CSF-1R$^{Tyr-708}$ peptide, and a K_m of 46 μM with the src$^{Tyr-527}$ peptide; in addition, the entire intracellular domain (D1 and D2) of the LRP (PTPα) PTPase also yielded similar results, with K_m values of 184 μM and 70 μM for these two peptides respectively (K. W. Harder and F. R. Jirik, unpublished work). Thus, the high K_m value observed when HPTPβ was tested on the CSF-1R$^{Tyr-708}$ peptide did not result from this peptide being a poor substrate for PTPases in general. This peptide, when present either in equimolar or 10-fold excess, did not inhibit the activity of HPTPβ on the src$^{Tyr-527}$ peptide (results not shown).

Much is known about the location and function of tyrosine phosphorylation sites within intracellular proteins. The insulin receptor β-chain, the epidermal growth factor receptor, and the kinase-insert regions of several other receptors PTKs, such as the PDGF and CSF-1 receptors, contain physiologically important phosphotyrosine residues that are found in close proximity to one another. For example, the kinase-insert region of PDGF-R contains three tyrosine phosphorylation sites within a stretch of 32 amino acid residues (Kashishian et al., 1992; Kazlauskas et al., 1992; Reedijk et al., 1992). The human insulin receptor β-chain (Ullrich et al., 1985) has three autophosphorylation sites at residues 1146, 1150 and 1151 (White et al., 1988; Flores-Riveros et al., 1989). These observations suggest that PTKs and PTPases

probably recognize substrate tyrosine and phosphotyrosine residues respectively, within the context of a short segment of primary sequence.

As the PTPases demonstrate great heterogeneity in their non-catalytic domains, it might be argued that the specificity of a given PTPase for its phosphoprotein substrate might be determined by protein–protein associations that depend on the non-catalytic regions of the PTPases. If correct, PTPase catalytic domains would be predicted to show a relative lack of specificity. As demonstrated by this study of HPTPβ, this appears not to be the case. In addition, there is other evidence of PTPases exhibiting substrate-specific differences in activity (Cho et al., 1991, 1992; Wang and Pallen, 1991; Ramachandran et al., 1992). In particular, a triphosphorylated synthetic peptide, derived from the insulin receptor β-chain sequence, has been used in a study using the PTPases CD45, LAR, T-cell PTPase and PTP 1B (Ramachandran et al., 1992). In these experiments, the PTPases differed with respect to the order with which they dephosphorylated the three phosphotyrosine residues in the peptide, illustrating differential PTPase substrate preferences within this 12-residue peptide sequence.

The kinetic analysis of HPTPβ provided further evidence that PTPases are capable of showing substrate-specific differences in activity when tested *in vitro*. HPTPβ demonstrated variations in affinity for the synthetic phosphopeptides selected, with the only variable between the different phosphopeptides tested being the composition of the sequences flanking the phosphotyrosine residue. It will be interesting to determine whether other PTPases exhibit similar differences in specificity when tested on this panel of synthetic phosphopeptide substrates. Such information may ultimately prove to be useful for predicting the substrate(s) of a given PTPase *in vivo*.

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