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Substrate Specificity of the Yeast Protein Tyrosine Phosphatase, PTP1, Overexpressed from an *Escherichia coli* Expression System.

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Abstract: A *Saccharomyces cerevisiae* Protein Tyrosine Phosphatase, PTP1, was expressed from an *Escherichia coli* expression system and milligram quantities of active PTP1 were purified chromatographically. The substrate specificity of the recombinant PTP1 was probed using synthetic phosphotyrosine-containing peptides corresponding to the regulatory phosphorylation sites of the yeast MAP kinase homologues $Fus3_{176-186}$, $Kss1_{179-189}$, and $Hog1_{170-180}$. Peptide sequences derived from the MAP kinase homologues were chosen arbitrarily as starting points for sequence variation studies even though they are not likely to be candidates for physiological substrates of PTP1. Phosphotyrosyl-$Hog1_{170-180}$ peptide showed a K_M value of 877 μM and phosphorylated $Kss1_{179-189}$ and $Fus3_{176-186}$ peptides showed lower K_M values of 74 μM and 51 μM each. To study the effect of sequence variations of the peptide, amino acids of the undecapeptide $Hog1_{170-180}$ (DPQMTGpY-VSTR) were sequentially substituted by an alanine residue. More extensive variations of each amino acid revealed positional importance of each amino acid residue. Based on these results, we derived a peptide sequence (DADEpYDA) that is recognized by PTP1 with an affinity (K_M is 4 μM) significantly higher than that of the peptides derived from the phosphorylation sites of Fus3, Kss1, and Hog1.
Key words: phosphotyrosyl peptide, protein tyrosine phosphatase, substrate specificity, yeast.

Protein tyrosine phosphatase (PTPase) is an enzyme that hydrolyzes the phosphoryl moiety from the phosphotyrosyl residue of cellular proteins (Walton and Dixon, 1993; Zhang and Dixon, 1994). In this sense, PTPases counteract the activities of protein tyrosine kinases (PTK), and the level of tyrosine phosphorylation within a cell is regulated by the balance of the activities of these two enzymes. Because the reversible tyrosine phosphorylation plays a crucial role in the regulation of various cellular processes including a variety of signal transduction processes, to understand the biological mechanisms of those processes, it is essential to comprehend the action of PTPase as well as PTK.

PTPases are generally distributed in eukaryotes, from human to yeasts, and dozens of PTPase genes have been cloned from various organisms. Extrapolating from the density of protein phosphatase genes in the chromosome, it was estimated that *C. elegans* could have about 230 protein phosphatase genes, half of them PTPases. From this, hundreds of PTPases genes could be expected to exist in humans (Hunter, 1995). From the unicellular eukaryote, *Saccharomyces cerevisiae*, two distantly related PTPase genes, PTP1 and PTP 2, have been cloned previously. Neither PTP1 nor PTP 2 was essential for growth, and disruption of PTP1 or PTP2 or both had no phenotypic change under vegetative growth conditions (Guan *et al.*, 1991, 1992). The physiological role of these yeast PTPases and their cellular substrates, however, largely remain to be elucidated, and only limited biochemical information is available. The function of PTP2 has recently been implicated in the two-component system that regulates an osmosensing MAP kinase pathway (Maeda *et al.*, 1994). Dephosphorylation of Hog1 MAP kinase was suggested as a possible role of PTP2. The recombinant PTP2, however, did not exhibit any tyrosine phosphohydrolase activities toward phosphorylated protein substrates *in vitro* (James *et al.*, 1992). The yeast *PTP1* gene was previously expressed in *Escherichia coli* and the recombinant protein PTP1 showed tyrosine-specific phosphohydrolase activities toward phosphorylated protein substrates (Guan *et al.*, 1991).

To delineate the biological roles of PTPases, one of the prerequisites is to understand how PTPases recognize their substrates. In many protein kinases, substrate specificity is largely controlled by the amino acid se-

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quences surrounding the phosphorylation sites (Kemp and Pearson, 1990; Kennelly and Krebs, 1991; Songyang *et al.*, 1995). It is not yet clear whether PTPases recognize its substrates mainly by the primary sequences around the tyrosine phosphorylation sites or by other factors. Nevertheless, the PTPases discriminate to a certain extent phosphotyrosyl peptide substrates with sequence variations. We and other laboratories have used chemically synthesized phosphotyrosine(pY)-containing peptides as substrates to probe the sequence-specific recognition of substrates by PTPases (Chatterjee *et al.*, 1992; Cho *et al.*, 1993, 1991; Hippen *et al.*, 1993; Ruzzene *et al.*, 1993; Zhang *et al.*, 1994, 1993). In those studies the effects of mutation of each amino acid residues around pY on the Michaelis-Menten kinetic parameters of PTPases were examined. In this way, amino acid residues critical for substrate recognition were derived for several PTPases.

In this study, we report the purification of the recombinant yeast PTPase, PTP1, and the optimal sequence context recognized by this enzyme, which was probed using synthetic phosphotyrosine-containing peptides. We studied the effects of sequence variations on three peptides whose sequences originated from the known tyrosine phosphorylation sites of cellular proteins. Extensive variations of each amino acid of the undecapeptides were made to learn the determinants for substrate recognition by PTP1. Based on these results, we deduced a peptide sequence that shows a K_M value which is significantly lower than those of the sequences adopted from the known phosphorylation sites.

Materials and Methods

Materials

Fmoc-amino acids including Fmoc-Tyr($PO_3{}^tBu_2$)-OH were purchased from AnaSpec (San Jose, USA). Fmoc-Tyr(PO_3Me_2)-OH, BOP, HOBT, Wang resin, IPTG, lysozyme, DNase, malachite green, Coomassie blue, buffer materials, Sephadex G-100, DEAE-Sephadex, CM-cellulose, and protein molecular weight marker were from Sigma (St. Louis, USA). Restriction enzymes and molecular biology products were from Promega (Madison, USA) or Boehringer Manheim (Manheim, Germany). Culture medium materials from Difco (Detroit, USA). All other chemicals and reagents were from Aldrich (Milwaukee, USA). Stirred cell and Amicon AM-30 protein concentrator were from Amicon (Beverly, USA). HPLC system was purchased from Orom tech (Seoul, Korea).

Buffers

Buffer A: 33 mM Tris-HCl, 2.5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM benzamidine, pH 8.0; Buffer B: 25 mM HEPES, 5 mM EDTA, 10 mM DTT, 1 mg/mL bovine serum albumin, pH 7.3; Buffer C: 100 mM HEPES, 10 mM DTT, 5 mM EDTA, pH 7.0; buffer D: 50 mM HEPES, 5 mM DTT, 2.5 mM EDTA, pH 7.0.

Synthesis of phosphotyrosyl peptides

The phosphotyrosyl peptides in Tables 2 were prepared by Fmoc solid-phase synthesis as described previously (Kitas *et al.*, 1991). Peptide syntheses were carried out manually in a 0.1 mmol scale and BOP, HOBT, and NMM were used as coupling reagents. Crude peptides were purified by reversed-phase HPLC (C18, 22 mm×250 mm, Alltech) with a linear gradient from H_2O/0.1% TFA to CH_3CN/0.1% TFA. The purified peptides were characterized by ion-spray mass spectroscopy.

Construction of pT7-7-PTP1

The plasmid pBlueskript SK(+)-PTP1 (kindly provided by Drs. H. Saito and T. Maeda) containing the yeast PTP1 gene was digested with *Hin*dIII. The 3700 bp fragment was separated and digested with *Pvu*II. The 2700 bp fragment thus generated was inserted into the SmaI site of pT7-7 prokaryotic expression vector. Thus, PTP1 lost three amino acids (MAA) from the amino-terminal and is fused to seven amino acids (MARIRAP). The first six amino acids (MARIRA) are derived from the pT7-7 vector and the proline residue from the junction of the vector and PTP1 coding sequence.

Overproduction of PTPase in *E. coli*

In order to overproduce the PTP1, the plasmid pT7-7-PTP1 was transformed into *E. coli* BL21(DE3), which carries an integrated copy of the T7 RNA polymerase gene under the control of the inducible *lac*UV5 promoter (Studier *et al.*, 1990). Addition of isopropyl β-D-thiogalactoside (IPTG) induces the production of T7 RNA polymerase in BL21(DE3), which in turn induces transcription of a sequence on the pT7-7 plasmid under the control of the T7 promoter.

Preparation of crude lysate was carried out as described previously (Tsai *et al.*, 1991). IPTG (final concentration, 0.4 mM) and 10% glucose (final concentration, 0.4%) were added to exponentially growing cultures of *E. coli* BL21(DE3) carrying the plasmid pT7-7-PTP1 in LB medium containing ampicillin (50 mg/mL). Cultures (3L) were shaken at 30℃ for 3 h, and cells were collected by centrifuging at 1000 g for 10 min at 4℃. The pellet was resuspended in 36 mL of buffer A (33 mM Tris-HCl, 2.5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM

benzamidine, pH 8.0). Lysozyme (5 mg) in 2 mL of buffer A was added, and the suspension was incubated at room temperature for 15 min followed by freezing in dry ice/ethanol. After being thawed with warm water, this was frozen and thawed for two more cycles. The resulting lysate was incubated with 200 μL of 1 M $MgCl_2$ and 200 μL of DNase (5 mg/mL in buffer A) for 30 min at 25℃. After addition of 0.8 mL of 0.5 M EDTA and 2.4 mL of 10% Triton X-100, the mixture was incubated at 25℃ for 10 min and transferred to two centrifuge tubes. This was centrifuged at 45,000 g for 30 min at 4℃ and the supernatant (46 mL) was stored frozen at −70℃.

Purification of PTPase

To the crude lysate obtained from 3L cultures of *E. coli* BL21(DE3)/pT7-7-PTP1 was added ammonium sulfate to a concentration of 50%, and the precipitate was isolated by centrifugation (10,000 g). The pellet was dissolved in 16 mL of buffer A (33 mM Tris-HCl, 2.5 mM EDTA, 10 mM β-mercaptoethanol, 1 mM benzamidine, pH 8.0) containing 0.5 M NaCl and applied to a gel filtration column (4.8 cm×52 cm, Sephadex G-100) equilibrated with buffer A containing 0.5 M NaCl. The column was then eluted with buffer A containing 0.5 M NaCl. The fractions containing PTPase activity (determined by the reaction with p-nitrophenyl phosphate as described in PTPase assay section) were pooled, concentrated to a volume of 5 mL (Stirred cell, Amicon), and dialyzed against buffer A. The PTPase sample was then applied to a DEAE-Sephadex column (1.6 cm×12 cm) equilibrated with buffer A. After washing with buffer A (50 mL), PTP1 was eluted with a linear salt gradient up to 1.0 M NaCl in buffer A. The active proteins elute with washing and/or at a very low NaCl concentration. The active fractions were pooled and concentrated to a volume of 2 mL (Amicon AM-30). It was loaded on a CM-cellulose column (1.5 cm× 6.7 cm), washed with buffer A (20 mL), and eluted with a NaCl gradient (0~1.0 M, 100 mL) in buffer A. The active fractions (ca. 0.25 M NaCl) were combined and concentrated (Amicon AM-30) to a volume of 1.5 mL (protein concentration of 6.4 mg/mL). Glycerol was added to the PTPase sample to a final concentration of 33% prior to being stored frozen at −70℃.

PTPase assay

To assay the dephosphorylation of phosphotyrosine containing peptides by PTP1, the release of inorganic phosphate was determined with the malachite green assay (Lanzetta *et al.*, 1979; Martin *et al.*, 1985). For this assay, the enzyme was diluted with buffer B (25 mM HEPES, 5 mM EDTA, 10 mM DTT, 1 mg/mL bovine serum albumin, pH 7.3) and added to the reaction mixture containing the phosphopeptide in buffer C (100 mM HEPES, 10 mM DTT, 5 mM EDTA, pH 7.0). Typically, total reaction volume was 50 μL after the addition of 5 μL (5~20 ng) of diluted enzyme, and the reaction mixture was incubated at 25℃ for 2~3 min. The assay was quenched with 0.9 mL of the malachite green reagent (Lanzetta *et al.*, 1979), and after 10 min at room temperature, the absorbance at 660 nm was determined. The nanomoles of Pi released was calculated by comparison to a standard curve determined for inorganic phosphate. For substrates with K_M values lower than 20 μM, total reaction volume was raised to 400 μL. In this case the reactions were carried out in buffer D (50 mM HEPES, 5 mM DTT, 2.5 mM EDTA, pH 7.0) in which the concentrations of components are one half of those in buffer C.

p-Nitrophenyl phosphate (pNPP) was used as a substrate for the purpose of PTPase purification and pH optimum measurement. In this case, the reaction conditions were exactly the same as described above except that pNPP (final concentration of 10 mM) was used as a substrate. The reaction was quenched by addition of 0.9 mL of 0.5 N NaOH solution and the absorbance was measured at 405 nm. p-Nitrophenol released was quantitated using a standard curve determined for p-nitrophenol.

Results and Discussion

Overproduction and purification of the PTP1 protein

In this study, PTP1 was expressed in *E. coli* expression system and purified chromatographically to homogeneity (Fig. 1). PTP1 was expressed under the control of a T7 promotor as described in Materials and Methods. Crude lysate was fractionated by ammonium sulfate precipitation followed by gel filtration chromatography to afford 8-fold purification. The protein was then subjected sequentially to DEAE-Sephadex anion exchange column chromatography and CM-cellulose cation exchange chromatography resulting in another 4-fold purification (Table 1). It required a total 40-fold purification and the purified 39 kDa PTP1 protein was apparently homogeneous as determined by polyacrylamide gel electrophoresis and Coomassie brilliant blue staining of the gel. Typically approximately 10 mg of PTP1 was obtained from 3L of *E. coli* culture.

Specific activity obtained with this protein was 31 μmol/min/mg toward pNPP at 10 mM pNPP concentration. PTP1 was overproduced previously by Guan *et al.* (1991) as a fusion protein with glutathione S-transferase. They reported specific activity of 27 μmol/min/mg toward pNPP which is a value very close to that

obtained in this study. Specific activities of 2~3 orders of magnitude lower were also reported toward Tyr-phosphorylated protein substrates such as myelin basic protein and Raytide. Phosphotyrosyl peptides used in this study, however, showed V_{max} values of 40~90 μmol/min/mg, higher than that toward pNPP.

pH optimum of PTP1

The recombinant PTP1 was active over a broad pH range, acidic to neutral, with a peak of activity at pH 7.0 toward pNPP as substrate. The enzyme activity decreased gradually as the pH became acidic, but the enzyme retained significant activity even below pH 4.0. The activity curve, however, showed a rapid decline above pH 7.5.

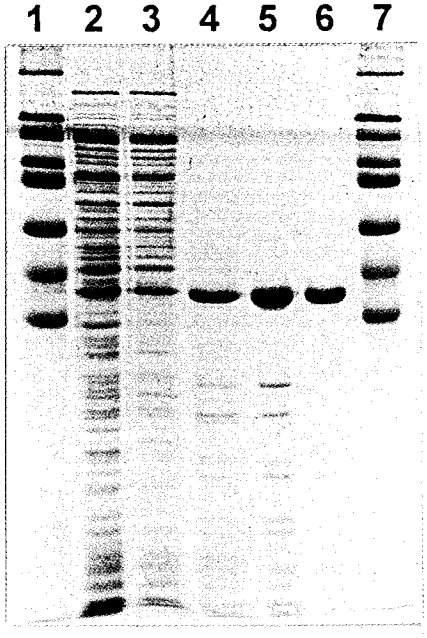

Fig. 1. SDS-PAGE analysis of PTP1 obtained from each step of PTP1 purification procedure. Lane 1 and 7, molecular weight standards, from top to bottom: 205, 116, 97, 84, 66, 55, 45, and 36 kD. Lane 2, crude cell lysate. Lane 3, 0~50% ammonium sulfate fraction. Lane 4, after gel filtration. Lane 5, after DEAE-Sephadex. Lane 6, after CM-cellulose. Protein samples were fractionated on a 10% SDS-polyacrylamide gel and visualized by staining with Coomassie brilliant blue.

Substrate specificity of PTP1 toward phosphotyrosine-containing peptides

To investigate the substrate specificity of the recombinant PTPase, PTP1, we used chemically synthesized phosphotyrosine-containing peptides. Initially tested as substrates were phosphotyrosyl(pY)-peptides corresponding to the amino acid sequences of the regulatory phosphorylation sites of yeast MAP kinase homologs, Fus3, Kss1, and Hog1 (Table 2, Line 1~3). Peptide sequences were the same as amino acid residues 176-186 of Fus3, 179-189 of Kss1, and 170-180 of Hog1 (designated as $Fus3_{176\text{-}186}$, $Kss1_{179\text{-}189}$, and $Hog1_{170\text{-}180}$ each) except that the tyrosine residue is phosphorylated in the peptide. These peptide sequences were chosen arbitrarily as starting points for sequence variation studies even though they are not likely to be candidates for physiological substrates of PTP1.

$Fus3_{176\text{-}186}$ and $Kss1_{179\text{-}189}$ showed K_M values of 51 μM and 74 μM each. $Fus3_{176\text{-}186}$ (QSGMTEpYVATR) and $Kss1_{179\text{-}189}$ (VGFMTEpYVATR) have exactly the same amino acids at positions −3 to +4 of the peptide, and the amino acid residues at −6, −5, and −4 positions, QSG and VGF, did not produce significant differencies in K_M values. $Hog1_{170\text{-}180}$ (DPQMTGpYVSTR) showed much higher K_M value of 877 μM. Amino acid sequence at positions +1 to +4 of $Hog1_{170\text{-}180}$ is similar to those at the same positions of $Fus3_{176\text{-}186}$ and $Kss1_{179\text{-}189}$; VATR *vs.* VSTR. Substitution of Ser with Ala in $Hog1_{170\text{-}180}$ resulted in exactly the same amino acid sequences as $Fus3_{176\text{-}186}$ and $Kss1_{179\text{-}189}$ on C-terminal side of pY. The resulting $Hog1_{170\text{-}180}$(S9A) has K_M value of 431 μM which is half of that of the parent peptide but still 6-8 fold higher than $Fus3_{176\text{-}186}$ and $Kss1_{179\text{-}189}$ (Table 2, Line 10). Differences in the

Table 1. Purification of the recombinant PTP1 from 3L of *E. coli* culture

	Volume (mL)	Concentration (mg/mL)	Total protein (mg)	Specific activity[a] (μmol/min/mg)	Total activity (μmol/min)
Crude lysate	46	18	828	0.75	621
0~50% $(NH_4)_2SO_4$	16	28	448	0.28[b]	125[b]
Gel filtration	42	1.2	50.4	8.3	418
DEAE-Sephadex	55	0.25	13.8	22.4	309
CM-cellulose	8	1.2	9.6	31	298

[a]Specific activity was measured using p-nitrophenyl phosphate (10 mM) as substrate as described in Material and Methods.
[b]For unknown reasons, reduced specific activity was obtained for 0~50% ammonium sulfate fractions. Total activity, however, was recovered after gel filtration.

Table 2. Kinetic data for PTP1 using phosphotyrosyl peptides as substrates

	Designations	Sequences	K_M (μM)	V_{max} (μmol/min/mg)
1	$Fus3_{176-186}$	QSGMTEpYVATR	51	80
2	$Kss1_{179-189}$	VGFMTEpYVATR	74	53
3	$Hog1_{170-180}$	DPQMTGpYVSTR	877	82
4	$Hog1_{170-180}$(P2A)	DAQMTGpYVSTR	284	80
5	$Hog1_{170-180}$(Q3A)	DPAMTGpYVSTR	417	71
6	$Hog1_{170-180}$(M4A)	DPQATGpYVSTR	463	66
7	$Hog1_{170-180}$(T5A)	DPQMAGpYVSTR	314	66
8	$Hog1_{170-180}$(G6A)	DPQMTApYVSTR	386	82
9	$Hog1_{170-180}$(V8A)	DPQMTGpYASTR	284	49
10	$Hog1_{170-180}$(S9A)	DPQMTGpYVATR	431	71
11	$Hog1_{170-180}$(T10A)	DPQMTGpYVSAR	599	84
12	$Hog1_{170-180}$(M4S)	DPQSTGpYVSTR	789	80
13	$Hog1_{170-180}$(T5E)	DPQMEGpYVSTR	265	80
14	$Hog1_{170-180}$(T5H)	DPQMHGpYVSTR	109	53
15	$Hog1_{170-180}$(G6E)	DPQMTEpYVSTR	76	77
16	$Hog1_{170-180}$(V8D)	DPQMTGpYDSTR	213	82
17	$Fus3_{177-186}$	SGMTEpYVATR	37	46
18	$Fus3_{177-185}$(M3A,T4H)	SGAHEpYVAT	40	45
19		ADEpYVAT	15	88
20		DADEpYVAT	7	42
21		DADEpYDAT	6	80
22		DADEpYDA	4	66

Amino acid residues mutated from the parent peptides were shown in bold type.

amino acid residues N-terminal side of pY of those peptides produced 6-8 fold differences of the affinities of the pY-peptides to PTP1 PTPase.

To investigate the effect of sequence variations on substrate recognition by PTP1, each amino acid at positions −5 to +3 of the undecapeptide $Hog1_{170-180}$ (DPQMTGpYVSTR) was substituted with an alanine residue one by one (Ala scan). This strategy was described previously by Zhang *et al.* (1993). Detailed kinetic data are shown in Table 2 (Line 4~11). In general general the Ala substitutions did not significantly change the K_M value probably because the substitutions did not change much the characteristics of the $Hog1_{170-180}$ peptide. The $Hog1_{170-180}$ sequence does not contain charged amino acid residues except D and R at both ends of the peptide. Ala substitutions for Pro of the $Hog1_{170-180}$ peptide lowered K_M value from 877 μM to 284 μM, a 3-fold change. Sequential Ala substitutions for Gln, Met, Thr, Gly, Val, Ser, and Thr residues of $Hog1_{170-180}$ peptide resulted in 1.5~3-fold decrease of K_M values suggesting that most of the amino acid residues in $Hog1_{170-180}$ peptide are not suitable choices for good affinity toward PTP1.

It is noteworthy that the amino acid residues at −3 and −2 positions are common in all three parent peptides, $Hog1_{170-180}$, $Fus3_{176-186}$, and $Kss1_{179-189}$; they are M and T. On the other hand, there is a noticeable difference in the amino acid at −1 position between those peptides; negatively charged residue, Glu, is common in $Fus3_{176-186}$ and $Kss1_{179-189}$, but it is Gly in $Hog1_{170-180}$. The difference at −1 position is considered meaningful, because the amino acid sequences at positions −6 to −4 of $Hog1_{170-180}$, $Fus3_{176-186}$, and $Kss1_{179-189}$ do not exhibit obvious differences and those at positions −3 and −2 are exactly the same. In this respect, it is not surprising that Gly to Glu mutation in $Hog1_{170-180}$ resulted in 12-fold decrease of K_M (from 877 μM to 76 μM) to a value close to those of $Fus3_{176-186}$ and $Kss1_{179-189}$ (Table 2, Line 15). This observation implies that Glu or acidic amino acid at −1 position is crucial for high affinity binding of peptide substrate to PTP1.

More extensive changes of amino acids at various positions were made to derive a optimum sequence recognized by PTP1 (Table 2, Line 12~16). M to S substitution at the −3 position did not produce a significant change in K_M value. M to A substitution at the same position lowered K_M (463 μM). At −2 position, T to E mutation resulted in 3-fold decrease in K_M value (265 μM). T to H mutation at the same position produced greater decrease of K_M (109 μM). These observa-

tions indicate that, at −2 position of the peptide, an amino acid residue with either a negatively or positively charged side chain provides higher affinity of the peptide substrates to the enzyme. Substitution of Val with Ala at +1 position resulted in a decrease in K_M value (284 μM). Asp is slightly better accomodated (K_M=213 μM) than Ala at the same position. At +1 position, therefore, hydrophobic residue is not good for high affinity toward PTP1, and an acidic residue is an amino acid of choice. In the Ala scan, we observed that Ser to Ala mutation ($Hog1_{170\text{-}180}$(S9A)) resulted in a 2-fold decrease of K_M (431 μM) and, Thr to Ala mutation ($Hog1_{170\text{-}180}$(T10A)), a small decrease of K_M (599 μM). Therefore, for both +2 and +3 positions, Ala substitution is better than those residues in wild type peptides.

$Fus3_{177\text{-}186}$, which is $Fus3_{176\text{-}186}$ minus N-terminal Q, showed higher affinity (K_M=37 μM) compared with the parent peptide (K_M=51 μM) implying that the amino acid residue at −6 position is not essential for high affinity substrate binding (Table 2, Line 17). There is also an indication that C-terminal Arg residue (+4 position) exerts a negative effect for the affinity toward PTP1 (data not shown). Based on the consideration that the two amino acids, Gln and Arg, at both ends of $Fus3_{176\text{-}186}$ are not necessary for high affinity substrate binding and the one that $Hog1_{170\text{-}180}$(T5H) showed 8-fold lower K_M value than $Hog1_{170\text{-}180}$, we tested a trial sequence SGAHEpYVAT which is a variant of $Fus3_{176\text{-}186}$; Gln and Arg at either ends deleted, Met at −3 position substituted with Ala, and Thr at −2 position substituted with His. The resulting $Fus3_{177\text{-}185}$ (M3A, T4H) produced almost the same K_M value (40 μM) as $Fus3_{177\text{-}186}$, the Q-deletion mutant of $Fus3_{176\text{-}186}$ (K_M=37 μM). This is an unexpected observation because only the C-terminal Q-deletion might increase the affinity, and the other two substitution mutations were also positive effectors. One possible explanation is that the presence of His at −2 position can produce either positive or negative effect depending on the sequences around His.

Based on these results, we tried to design a peptide sequence that has higher affinity toward PTP1 than the previously tested ones (Table 2, Line 19~22). Because a negatively charged amino acid residue at either −1 or −2 position was advantageous, we introduced negatively charged amino acids at both of these positions. The trial peptide ADEpYVAT showed K_M value of 15 μM, a value significantly lower than those obtained for any other peptides tested thus far. This sequence is quite similar to the optimum sequences recognized by *Yersinia* PTPase and rat PTP1 (Zhang *et al.*, 1994). For both enzymes, the consensus substrate was DADEpYL-NH_2. It is noteworthy that Asp is present at −4 position of the peptide. Because we have not tried Asp substitution at −4 position of parent peptides, we tested negative charge option to the peptide ADEpYVAT. When Asp was added to the N-terminal end of the latter, the resulting octamer DADEpYVAT produced a 2-fold decrease of K_M value (7 μM). Therefore, DADE is considered as a good consensus sequence for the N-terminal side of pTyr residue.

As for the C-terminal side of pY, we noted above that substitution of Val with Asp at +1 position of $Hog1_{170\text{-}180}$ decreased K_M value (from 789 μM to 213 μM). When Asp was substituted for Val at +1 position. The resulting octamer, DADEpYDAT, again produced a decrease of K_M value (6 μM). This is in contrast with *Yersinia* PTPase and rat PTP1 in that, for those enzymes, the negative charge on the carboxylate at the +1 position is detrimental for PTPase recognition. It is not yet clear that Thr at +3 position is essential to maintain the affinity, therefore, we tested a shorter version, DADEpYDA. This peptide produced lower K_M value value of 4 μM proving that Thr residue at −3 position is not necessary.

The sequence, DADEpYDA, recognized efficiently by yeast PTP1 PTPase has acidic residues at the positions −4, −2, −1, and +1 relative to phosphotyrosine. Similar trends were also found in many other PTPases. T-cell PTPase (TC-PTP) showed preferences for acidic residues at positions −4, −3, and −1 (Ruzzene *et al.*, 1993). The consensus sequences for PTP-5 from bovine brain and PTP-1 from rat brain also have similar trends (Hippen *et al.*, 1993). HPTPβ exhibited a preference for Glu or Asp at positions −4 to −1 (Cho *et al.*, 1993). PTP1B from human placenta also prefers acidic amino acids at −1 and −2 positions (Zhang *et al.*, 1993) and, in this case, the sequence specificity was explained on the molecular basis by Jia *et al.* (Jia *et al.*, 1995). X-ray crystal structure of inactive PTP1B complexed with a phosphotyrosyl peptide revealed that Arg47 of PTP1B forms salt bridges with the carboxyl side chains of the amino acids at −1 and −2 positions of the peptide. Jia *et al.* (1995) also indicated that rat brain PTP-1, *Yersinia* PTP, and TC-PTP, which share a preference for peptides with acidic amino acid residues N-terminal to pTyr, all contain Arg at the equivalent position of Arg47 of PTP1B. Yeast PTP1 in this study, on the other hand, has Val, not Arg, at the position but still exhibits sequence specificity similar to the PTPases mentioned above. HPTPβ does not have Arg at the equivalent position, either. It is Gln in HPTPβ (Kreuger *et al.*, 1990). Diverse amino acid residues appear in PTPases at the position equivalent to Arg47 of PTP1B; they are Ser, Lys, Val, Ile, Gln, Thr, Ala, and Arg. The structure-specificity relation-

ship to be explained unambiguously requires more extensive studies on the substrate specificities of PTPases as well as accumulation of the structural data of more PTPases complexed with diverse substrates.

Yeast PTP1, together with the PTPases mentioned above, constitute a class of PTPases that show higher affinity toward peptides with acidic amino acid residues on the N-terminal side of pTyr. Even though subcellular localization of PTPases can be suggested as one of the major determinants for *in vivo* substrate specificities of PTPases, there exists differences between PTPases in the recognition of primary sequences of peptide substrates. Information on this differential recognition can be invaluably used for the design of inhibitors and for the study of the physiological role of PTPases.

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