A Single Amino Acid Substitution Alters Substrate Sequence Specificity of the Yeast Protein Tyrosine Phosphatase YPTP1

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Protein tyrosine phosphatases (PTPases) constitute a family of enzymes that catalyze the hydrolysis of a phosphate moiety from a phosphotyrosyl residue of a protein.[1-5] Because of the biological importance of the reversible tyrosine phosphorylation, much research has been focused on the PTPases since its first identification in 1988.[6] More than 70 PTPases have been identified in various organisms including human to yeasts.[7] Much progress has been achieved in the study of the mechanism of catalysis.[4] However, *in vivo* substrates and biological roles of most of the PTPases still remains to be elucidated.

PTPases recognize short phosphotyrosyl(pY)-peptides as substrates and the sequence specificities of several PTPases toward pY-peptides have been investigated.[7-13] We previously reported that the lowest K_M substrate for the yeast PTPase, YPTP1, was DADEpYDA which is characterized by the multiple acidic residues at the N-terminal side of pY.[8] Similar trends were also found in many other PTPases including PTP1B,[9] HPTPβ,[10] T-cell PTPase,[11] PTP-5 from bovine brain and PTP-1 from rat brain.[12] *Yersinia* PTPase and rat PTP1.[13]

In the case of PTP1B, the sequence specificity was explained on the molecular basis by Jia *et al.*[14] X-ray crystallographic analysis of inactive PTP1B complexed with a phosphotyrosyl peptide, DADEpYL-NH_2, revealed that Arg-47 is responsible for defining the sequence specificity of PTP1B; Arg-47 forms salt bridges with the carboxyl side chains of Asp and Glu at -1 and -2 position of N-terminal side of pY (pY-1 and pY-2 positions) of the peptide. It is noteworthy that rat brain PTP-1, *Yersinia* PTPase, and T-cell PTPase, which share a preference for pY-peptides with acidic amino acid residues N-terminal to the pY, contain Arg at the equivalent position of Arg-47 of PTP1B.[15,16]

YPTP1, on the other hand, has Val-59,[17] not Arg, at the equivalent position still exhibiting sequence specificity similar to the PTPases mentioned above. HPTPβ does not have Arg at the position, either. It is Gln in HPTPβ.[15] Diverse amino acid residues appear in PTPases at the position equivalent to Arg-47 of PTP1B; they are Ser, Lys, Val, Ile, Gln, Thr and Ala.[5]

To investigate if Val-59 is an important determinant for the peptide substrate recognition by YPTP1, we substituted Val-59 with Asp or His by site-directed mutageneses and performed kinetic experiments. These mutations that change the hydrophobic side chain of Val to amino acid side chains with a negative or a positive charge might alter the preferences of YPTP1 for the peptide substrate sequences. Previously we subcloned the *YPTP1* gene in the *E. coli* expression vector pT7-7 (pT7-*YPTP1*) and used it for the overexpression of the wild-type YPTP1.[8] To generate the mutants, YPTP1 (V59D) and YPTP1 (V59H), we modified pT7-*YPTP1* plasmid by the "overlap extension method"[18] based on polymerase chain reaction (PCR) technique. The wild-type and mutant PTPases were purified to > 90% purity by the combination of affinity chromatography and conventional chromatography techniques as described previously for wild-type YPTP1.[8,19,20] A few milligrams of wild-type or mutant enzymes are generally obtained from 1 liter of *E. coli* culture. Specific activities of purified mutant enzymes toward p-nitrophenyl phosphate are 30-40 μmol $\cdot$ $min^{-1}mg^{-1}$ which are essentially indistinguishable with that of wild-type YPTP1.[10,19]

Kinetic experiments were performed with wild-type and the two mutant phosphatases (Table 1).[8] Chemically synthesized 5 mer pY-peptides with sequences AXApYA (X is a variable amino acid) were used as substrates.[10,21] Eventhough the negatively charged residues at pY-2 and pY-1 positions are both necessary for high affinity recognition by YPTP1, to avoid complication, the -1 position is fixed to Ala and only the -2 position of pY was changed sequentially.

The first substrate examined in this study was ADApYA. Toward this substrate wild-type YPTP1 exhibited a K_M value of 34 μM, 8.5-fold higher than that of the lowest K_M substrate DADEpYDA (K_M = 4 μM).[8] Because of the importance of the negative charge at pY-2 position of the peptide, it is anticipated that the introduction of a positively charged amino acid to that position would reduce the affinity for wild-type YPTP1 and this was truely the case: K_M = 159 μM

Table 1. K_M values with Wild-Type and Mutant Recombinant Yeast YPTP1 (unit: μM)

Line	Peptides	YPTP1		
		wild-type	V59D mutant	V59H mutant
1	H_2N-ADApYA-OH	34	220	280
2	H_2N-ARApYA-OH	159	92	290
3	Ac-ADApYA-NH_2	43	162	198
4	Ac-AHApYA-NH_2	156	127	> 400

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for ARApYA (Line 2).

To determine if Val-59 is an important determinant for the recognition of the pY-2 residue of peptide substrates, we substituted Val-59 of YPTP1 with the negatively charged Asp residue. YPTP1(V59D) mutant showed 7-fold reduced affinity toward ADApYA (Line 1) and 2-fold increased affinity toward ARApYA (Line 2). Collectively, ADApYA was a better substrate for wild-type YPTP1 and ARApYA was better for the mutant enzyme.

When the N-termini of the peptides were acetylated and the C-termini were amidized to eliminate the effect of the charge at both ends, the peptide substrates showed similar trends. Ac-ADApYA-NH_2 gave K_M values not much different from those obtained with the unmodified version of the peptide with the same sequence for the wild-type and mutant enzymes (Line 1 and 3). Results obtained with Ac-AHApYA-NH_2 (Line 4) was also comparable with those obtained with ARApYA (Line 2). These results implicate that Val-59 of YPTP1 is involved in the recognition the pY-2 residue of the pY-peptide substrate.

Substitution of Val-59 with His in YPTP1, in agreement with the results described above, was not favorable for the interaction of the enzyme with Ac-AHApYA-NH_2 (Line 4). YPTP1(V59H), however, did not differentiate Asp and Arg at pY-2 position of the peptide (Line 1 and 2). This result, together with the fact that V59H mutant exhibits low affinity toward any substrates tested, indicates that electronic interaction is not a prevailing factor in this case.

It may be possible to speculate that the peptide substrates bind to YPTP1 in a peptide sequence-dependent manner; *i.e.* positively charged Arg residue of H_2N-ARApYA-OH interacts favorably with the negatively charged Asp-59 residue of YPTP1(V59D) thereby exhibiting a low K_M; Asp residue of H_2N-ADApYA-OH, however, interacts with a residue other than Val-59 of YPTP1 for high affinity binding to wild-type YPTP1. We constructed a plasmid encoding V59R mutant for extended study but unfortunately this mutant protein is not expressed in an active form in *E. coli*. Unambiguous explanation of the structure-specificity relationship of YPTP1 would be possible by extensive studies of X-ray crystal structures of YPTP1 and its mutants complexed with pY-peptides, and these studies are in progress.

In summary, we modified the sequence specificity of YPTP1 for the pY-peptide substrates by the single amino acid substitution. Especially V59D mutation reversed the specificity of the wild-type enzyme. This study also demonstrates that Val59 plays an important role in the recognition of the pY-peptide substrates.

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Construction of Val59 → Asp Mutant Gene of YPTP1

The Val-59 to Asp mutation utilized four oligonucleotide primers (CH-44, 46, 50, and 52). CH-44 is a mismatched primer in which the codon GTT for Val is converted to GAT for Asp. CH-50 has the complementary sequence of CH-44. CH-46 and CH-52 are flanking primers 1240 and 1040 base pairs away from the mutation site in pT7-*YPTP1*.

Three rounds of PCR was performed to prepare the mutant construct. The first round of PCR was performed using CH-44 and CH-46 with pT7-*PTP1* plasmid as a template to obtain the PCR product 1. The second round of PCR utilizing primers CH-50 and CH-52 produced the PCR product 2. The third round of PCR utilized the double-stranded PCR products 1 and 2 as templates and CH-46 and CH-52 as primers and the amplified PCR product was ligated with pCR™2.1 vector (Promega). The ligation product was then digested with Ksp I and Nco I and the 715bp fragment was inserted into the Ksp I / Nco I site of pT7-*PTP1* to complete the construction. The MluI/PstI fragment of the mutant construct was then replaced with the same part of wild-type pT7-*PTP1* to avoid any undesired mutation. Primers: CH-44 (5'-gCT AgA AAC AgA TAC gAT AAC ATT ATg CCg TAT gAg-3', nucleoties 981-1016 of *YPTP1* gene), CH-46 (5'-CgA TAC CgA TAg CAC CTT ggA A-3', antisense of nucleoties 2200-2221 of *YPTP1*), CH-50(5'-CTC ATA Cgg CAT AAT gTT ATC gTA TCT gTT TCT AgC-3'), CH-52(5'-gAg CgA ACg ACC TAC ACC gAA C-3', nucleoties 896-917 of pT7-7 vector).

Construction of Val59 → His Mutant Gene of YPTP1

The same strategy was used to prepare the construct for the V5911 mutant except that the mutant primers CH-56 and CH-57 were used instead of CH-44 and CH-50. In CH-56, the codon GTT for Val is replaced with CAT for Asp. Flanking primers were the same. Primers: CH-56 (5'-C gAT gCT AgA AAC AgA TAC CAT AAC ATT ATg CCg TAT gAg-3'), CH-57 (5'-CTC ATA Cgg CAT AAT gTT ATg gTA TCT gTT TCT AgC ATC g-3').

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