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Research Communication

Tyr306 Near the C-terminus of Protein Phosphatase-1 Affects Enzyme Stability and Inhibitor Binding

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Summary

Previous deletion studies have suggested that Tyr306 has an important influence on the properties of protein phosphatase-1 (PP1). To test this inference, we constructed three site-directed mutants, PP1Y306A, PP1Y306K, and PP1Y306E. The specific activity of PP1Y306A was 3.5-fold higher than that of PP1wt, especially with K-R-Tp-I-R-R as substrate, and it also had a 13-fold higher K_{cat} and a 43-fold higher K_{cat}/K_m. PP1Y306K and PP1Y306E, in that order, and were next in terms of increased activity. Use of the denaturant guanidine hydrochloride (GdnHCl) demonstrated that mutation of this site decreased enzyme stability. PP1Y306A and PP1Y306E lost all activity when incubated for 24 h in 0.6 M GdnHCl, and their fluorescence spectra confirmed the loss of stability. Because all three substitutions had a similar effect, we infer that the aromatic group of Tyr plays a crucial role in maintaining enzyme stability. Our results show that Tyr306 does affect the spatial conformation of the catalytic subunit of PP1 molecule. The IC_{50} of PP1Y306A for the inhibitor microcystin-LR was threefold higher than that of PP1wt, whereas those of PP1Y306E for tautomycin and norcantharidin were 15-fold and 10-fold higher, respectively. We conclude that Tyr306 plays an important role in enzyme stability and inhibitor binding.

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Keywords protein phosphatase-1; Tyr306; stability; inhibitor; inhibition.

Abbreviations IC50, the half maximal inhibitory concentration; HIV-1, human immunodeficiency virus-1; PPP, protein phosphatase P; pNPP, p-Nitrophenyl phosphate; IPTG, isopropyl-β-D–galactoside; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; EGTA, ethyleneglycol-bis(β-aminothyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MOPS, 3-(N-Morpholino) propanesulfonic; DTT, dithiothreitol.

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INTRODUCTION

Protein phosphatase-1 (PP1) belongs to the highly conserved PPP family of Ser/Thr protein phosphatases (*1, 2*). The family comprises PP1, PP2A, PP2B, PP4, PP5, PP6, and PP7. PP1 regulates many cellular processes, such as cell cycle progression, protein synthesis, muscle contraction, glycogen metabolism, transcription, and neuronal signaling. It is also an important regulator of HIV-1 transcription due to its interaction with Tat, and hence a potential target of anti-HIV-1 therapy (*3*). In addition, PP1 plays important role in DNA repair (*4*). It is a heterodimer composed of a fixed catalytic subunit and one of dozens of different regulator subunits. The catalytic subunit is localized in different regions of the cell by the different regulatory subunits, and so can regulate different processes. The substrate specificity and diversity of PP1 is mainly determined by its regulatory subunits (*5, 6*).

Research on the catalytic subunit of PP1 (PP1c) has mainly focused on the effects of inhibitors (*7, 8*). Microcystin-LR (MCLR), tautomycin (TM), okadaic acid (OA), and norcantharidin (NCTD) are among the most thoroughly investigated inhibitors and have been used to dissect the roles of the individual phosphatases in signaling pathways (*9–11*) (Fig. 1). MCLR is a hepatotoxic cyclic peptide associated with most strains of the blue-green alga *Microcystis aeruginosa* found in the Northern hemisphere. It is the most investigated cyclic peptide toxin, with a ring structure formed by seven amino acids, and a potent inhibitor of PP1 and PP2A (*10, 12, 13*). OA is a tumor-promoting C38 polyether fatty acid produced by marine dinoflagellates. It contains acidic and hydrophobic moieties and is cyclized (via an intramolecular hydrogen bond) (*11*). This toxin can accumulate in filter-feeding organisms and is the principal cause of diarrhetic shellfish poisoning worldwide (*14*). TM is similar to

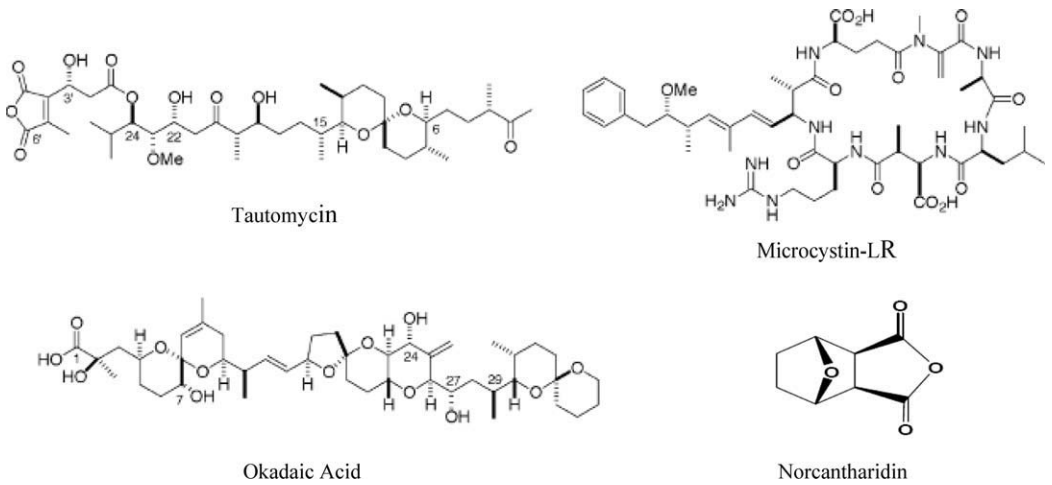

Figure 1. Structures of the natural product inhibitors of PP1.

a part of the OA molecule, and it is a potent inhibitor of PP1 and PP2A and a potent tumor promoter. It exists in solution as an equilibrium between two tautomers: a diacid and an anhydride, and the form that inhibits PP1 is the diacid. The TM molecule can be divided into two functional fragments: 1) a hydrophilic moiety that contains the tautomeric center in the diacid form and binds the active site of PP1, and 2) a hydrophobic bicyclic ketal moiety that forms contacts with the hydrophobic groove of PP1 (*15, 16*). Cantharidin is also a powerful inhibitor of the PPP family of phosphatases, with severe effects on the gastrointestinal tract, kidney, and ureter (*17, 18*). Cantharidin and its demethylated form, NCTD, are also effective against multidrug-resistant leukemia, hepatoma, and myeloma cells. NCTD is less toxic than cantharidin and has been used in China since the 1980s to treat human cancers (*19*).

The emphasis of inhibition studies has been mainly on defining the conserved domains of the catalytic subunit, whereas the function of the nonconserved termini in binding these inhibitors and in enzyme catalysis has been little investigated. It appeared likely to us that the nonconserved regions were responsible for the differences between the various protein phosphatases and that study of these regions might help to explain why the various protein phosphatases catalyze different reactions despite having high sequence identities. Although the regulatory subunits of protein serine/threonine phosphatases are important in determining substrate specificity and catalytic activity, we considered the possibility that the nonconserved regions of the catalytic subunit might also play a role. In particular, previous work has suggested that Tyr306 of PP1 is functionally important.

In this study, we constructed three site-directed mutants of PP1 Tyr306, PP1Y306A, PP1Y306K, and PP1Y306E, to examine the importance of this residue. We found that substitutions at this site led to increased substrate affinity and reduced stability as well as higher IC_{50} values for MCLR, TM, and NCTD. The latter observation suggests that this site is involved in the interaction with these inhibitors.

MATERIALS AND METHODS

Materials

OA, MCLR, NCTD, PNPP, IPTG, Tris, EDTA, $MnCl_2$, and $CaCl_2$ were purchased from Sigma Chemical Corporation. Substrates (K-R-Tp-I-R-R) and RII were synthesized by Scilight Biotechnology, LLC (China). The PP1 plasmid, pTACTAC expression vector, and strain DH5α were from our laboratory. Restriction enzymes (NdeI and Hind III), T4 DNA ligase, and Pyrobest polymerase were purchased from TaKaRa Biotech (Tokyo, Japan). Malachite green and TM were from BIOMOL International, LP. All other reagents used were of analytical grade. The PP1 mutants were all based on rabbit muscle PP1α isoform cDNA.

Construction of Site-Directed Mutants of PPase-1 in pTACTAC Vector

Site-directed mutants of PP1 were constructed by PCR, and then inserted into pTACTAC vector. The site-directed mutants were PP1Y306A (Tyr306 changed to Ala), PP1Y306K (Tyr306 changed to Lys), and PP1Y306E (Tyr306 changed to Glu). The 5′ and 3′ end primers for PP1Y306A, PP1Y306K, and PP1Y306E were the same as for PP1 wild type; the 5′ primer was 5′ TATA CAT ATG TCC GAC AGC GAG AAG CTC 3′ (Nde I site underlined), and the 3′ primer was 5′ GC AAG CTT CTA TTT CTT GGC TTT GGC A 3′ (Hind III site underlined). The linking primers for PP1Y306A were 5′ GAA CTG CCC CGC CTT GCC CTT GTT 3′ and 5′ AG GGC AAG GCG GGG CAG TTC AGT 3′ (substituted nucleotides are underlined). The linking primers for PP1Y306K were 5′ GAA CTG CCC TTT CTT GCC CTT GTT 3′ and 5′ AG GGC AAG AAA GGG CAG TTC AGT 3′, and for PP1Y306E, they were 5′ GAA CTG CCC TTC CTT GCC CTT GTT 3′ and 5′ AG GGC AAG GAA GGG CAG TTC AGT 3′. The PCR products were digested with *Nde*I and *Hind* III and ligated into pTACTAC, then transformed into *Escherichia coli* DH5α.

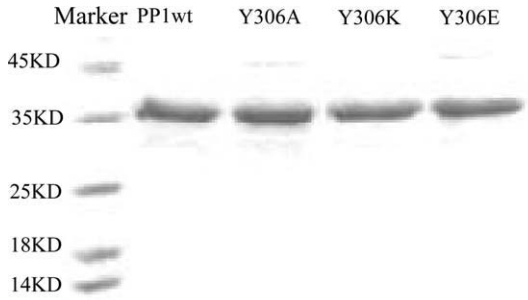

Figure 2. SDS-PAGE electrophoresis of PP1 and the site-directed mutants PP1wt, PP1Y306A, PP1Y306K and PP1Y306E from left to right.

Expression and Purification of Proteins

Expression and purification were performed as for PP1 (*7*). Enzyme purity was assessed by 12% SDS-PAG, and protein concentration was measured by the method of Bradford.

Protein Phosphatase Activity and Inhibition Assays

Assay reactions were carried out by mixing 25 μl Ser/Thr assay buffer (20 mM MOPS, pH 7.5, 1 mM DTT, 0.15 M NaCl, 0.1 mM $MnCl_2$, 1 mM $MgCl_2$, 2 mM EGTA, 10% glycerol, and 0.01 mg/ml serum albumin), 15 μl distilled water, and 5 μl of aqueous diluted substrate (K-R-Tp-I-R-R), incubating at 30 °C for 10 min, immediately adding 5 μl diluted recombinant enzyme in enzyme dilution buffer, 50 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 1 mM DTT, and 1 mg/ml bovine serum albumin, and incubating further at 30 °C for 30 min (*20*). A total of 100-μl malachite green solution was then added to each reaction, and after incubation for 15 min, phosphate release was quantified by measuring absorbance at 630 nm in a microtiter plate reader. All reactions were performed in triplicate. One unit of phosphatase activity was defined as the amount of enzyme required to hydrolyze 1 nmol of Pi in 1 min at 30 °C in a total reaction volume of 50 μl.

Assay reactions with PNPP as substrate were performed according to (*9*).

Inhibition reaction was assayed with K-R-Tp-I-R-R was substrates. Inhibition assays were performed in the same way except that the 15 μl of distilled water was replaced with MCLR, TM, NCTD or OA solutions of different concentrations. In addition, the inhibitors were incubated with enzyme for 5 min at 30 °C before addition of substrate.

Fluorescence Spectroscopy

Fluorescence measurements were performed using a FLOR-MAX-2 fluorometer. Suspensions of PP1wt and the mutants (5 μM) in buffer were incubated with various concentrations of guanidine hydrochloride (GdnHCl) at 4 °C for 24 h to reach chemical and thermal equilibrium. The samples were excited at 295 nm using 5 nm excitation and emission slit widths, and intrinsic fluorescence emission spectra were recorded between 310 nm and 410 nm.

RESULTS

Protein Phosphatase Activity and Kinetic Parameters

We constructed three site-directed mutants, PP1Y306A, PP1Y306K, and PP1Y306E, to assess the importance of Tyr306. All of the enzymes were expressed in *E. coli* and purified to near homogeneity (Fig. 2). We selected PNPP and K-R-Tp-I-R-R as substrates to measure enzymatic activity. We found no obvious changes of the enzyme activities of PP1Y306K and PP1Y306E with PNPP as substrate. In the case of PP1Y306A, activity increased from 10,622 ± 317 U/mg to 16,061 ± 673 U/mg. With K-R-Tp-I-R-R as substrate, its activity increased from 953 ± 26 U/mg to 3,325 ± 103 U/mg and the activities of the other two mutants also obviously increased, though somewhat less (Fig. 3).

The results of a kinetic analysis of these mutants with K-R-Tp-I-R-R as substrate are presented in Table 1. When Tyr306 was substituted by Ala, the K_m for K-R-Tp-I-R-R decreased from 385 ± 19.4 μmol to 116 ± 4.8 μmol, and K_{cat}/K_m increased from 623 ± 14.6 mol^{-1} L sec^{-1} to 27,021 ± 29.1 mol^{-1} L sec^{-1}, a 43-fold increase. The K_{cat} of Y306A also increased from 0.24 ± 0.13 sec^{-1} to 3.14 ± 0.25 sec^{-1}, whereas the K_{cat}'s of PP1Y306K and PP1Y306E increased slightly less.

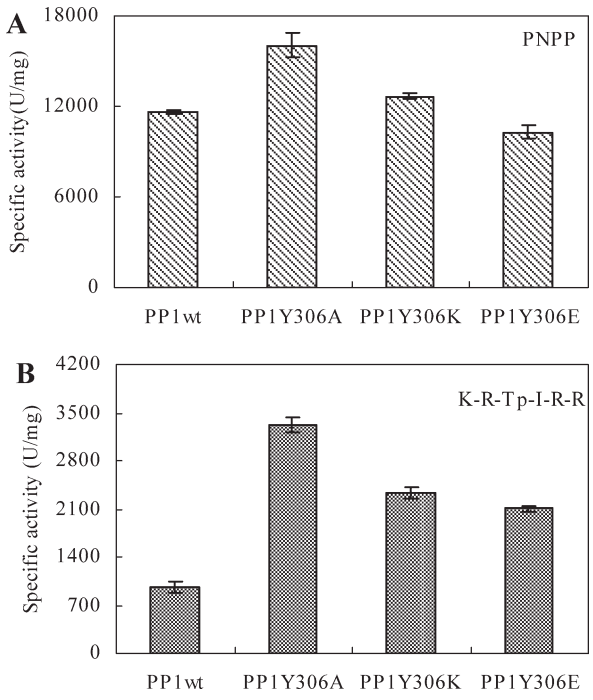

Figure 3. Specific activities of PP1wt and the mutants. A: Purified enzymes assayed with PNPP as substrate; B: Purified enzymes assayed with K-R-Tp-I-R-R as substrate. The assays were performed as described in Materials and Methods.

Table 1
Kinetic analysis of mutants[a]

Enzyme	K_m	K_{cat}	K_{cat}/K_m	V_{max}
PP1wt	385 ± 19.4	0.24 ± 0.13	623 ± 14.6	799 ± 21.3
PP1Y306A	116 ± 4.8	3.14 ± 0.25	27,021 ± 29.1	1,630 ± 17.4
PP1Y306K	171 ± 7.9	3.01 ± 0.27	17,518 ± 19.4	1,250 ± 14.8
PP1Y306E	111 ± 12.1	2.07 ± 0.12	18,665 ± 5.8	1,340 ± 20.7

[a]Kinetic analyses of the mutants were performed as described in Materials and Methods section. K_m values for K-R-Tp-I-R-R are in micromolar. K_{cat} values are sec^{-1}. Values for K_{cat}/K_m are in mol^{-1} L sec^{-1}. V_{max} values is μmol L^{-1} min^{-1}.

Stability of the Mutants

The stability of the three mutants in the denaturant GdnHCl was clearly lower than that of PP1wt (Fig. 4). After incubation in 0.4 M GdnHCl, PP1Y306A and PP1Y306E retained only about 20% residual activity, whereas PP1Y306K was somewhat more stable. After incubation in 0.6 M GdnHCl mutants, only PP1Y306K retained any activity. Clearly, Tyr306 plays an important role in the stability of the PP1c molecule.

Fluorescence Spectra of the Mutants in Different Concentrations of GdnHCl

We also examined changes in the spatial conformation of the mutant enzymes after 24 h in various concentrations of GdnHCl by studying their intrinsic fluorescence spectra. In 0.6 M GdnHCl, the emission peaks of PP1Y306K and PP1Y306E, which lost all activity, were red shifted by 10 nm and 13 nm, respectively (Figs. 5B and 5C), and much the same was true of PP1Y306A (data not shown). At the same time, the emission peak of the wild type did not change; there was only a slight fall in its fluorescence intensity. In 2 M GdnHCl, the fluorescence intensity of the mutants increased, and their emission peaks were red shifted even more as the high GdnHCl concentration completely destroyed the conformation of the molecules, the peptide chains were stretched, and Trp and Tyr residues normally buried internally were transposed from a nonpolar to a polar environment. Meantime, the emission peak of PP1wt was only red shifted by 3 nm. These data further show that Tyr306 influences enzyme stability.

Inhibition of the Phosphatases

We examined inhibition of the mutant enzymes by MCLR (Fig. 6A, Table 2). The sensitivity of PP1Y306A to MCLR was lower than that of the wild type enzyme; the IC_{50} for PP1Y306A was 53.0 ± 2.2 nM, and that of PP1wt, approximately 18.0 ± 1.1 nM. When Tyr306 was mutated to Glu, IC_{50} increased to 28.9 ± 2.2 nM.

The sensitivity of the PP1Y306A mutant to inhibition by TM was little changed, and its IC_{50} values was 35.1 ± 2.3 nM, which increased 1.3-fold than that of PP1wt (IC_{50} 28.2 ± 1.5 nM), whereas the sensitivity of the Lys- and Glu-substituted enzymes was appreciably reduced (Fig. 6B, Table 2); the IC_{50} of PP1Y306K (292.9 ± 10.5 nM) and PP1Y306E (397.8 ± 5.1 nM) increased 10-fold and 15-fold, respectively.

We also examined inhibition of these mutants by OA (Fig. 6C, Table 2). PP1Y306K (IC_{50} 1.2 ± 0.1 μM) and PP1Y306E (IC_{50} 1.7 ± 0.2 μM) were more sensitive than wild type (IC_{50} 3.6 ± 0.1 μM), whereas PP1Y306A (IC_{50} 6.3 ± 0.2 μM) was 1.8-fold less sensitive. The sensitivity of all three mutants to NCTD was reduced (Fig. 6D, Table 2). Mutation to Glu-reduced sensitivity was about 10-fold (IC_{50} 333.0 ± 8.9 μM), and the sensitivity of the other two mutants was reduced by 1.5-fold to 5-fold.

DISCUSSION

We found in unpublished experiments that deletion of 24 amino acid at the C-terminus of PP1 (PP1-(1-306)) increased enzyme activity from 953 ± 26 U/mg to 3,368 ± 124 U/mg with K-R-Tp-I-R-R as substrate, whereas others have reported that deletion of the 25 C-terminal amino acids reduced activity

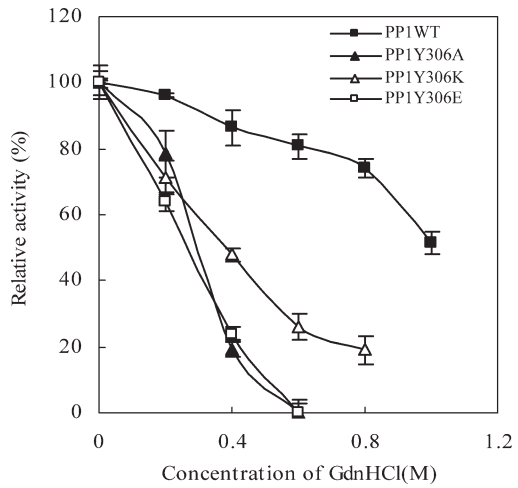

Figure 4. Stabilities of PP1wt and mutants in the denaturant GdnHCl. Purified PP1wt (■), PP1Y306A (▲), PP1Y306K (△), PP1Y306E (□) were assayed with PNPP as substrate. Different concentrations of GdnHCl were incubated with the enzymes (5 μM) at 4°C for 24 hours.

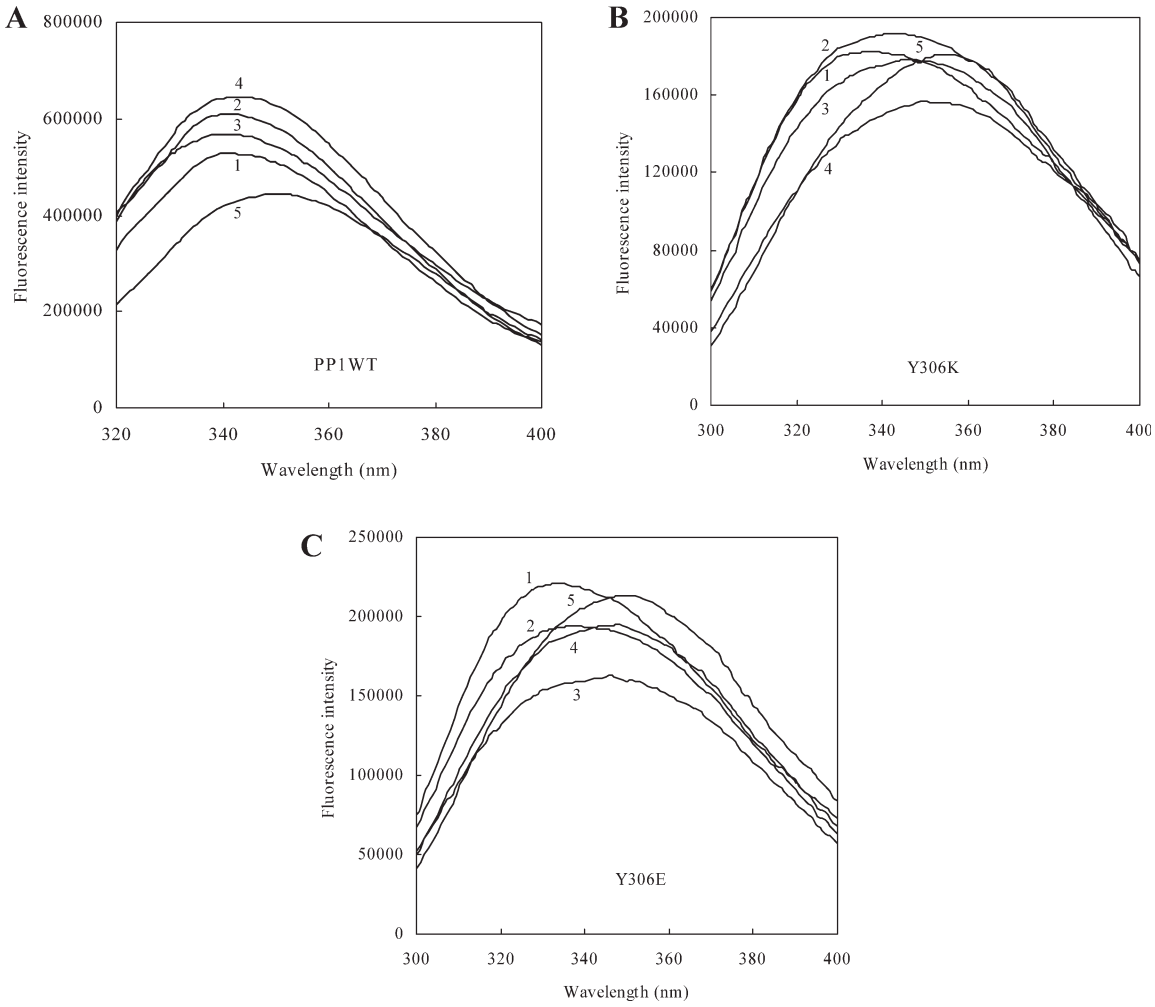

Figure 5. GdnHCl-induced changes in the intrinsic fluorescence spectra of PP1wt and the mutants. Different concentrations of GdnHCl were incubated with the enzymes (5 μM) at 4°C for 24 hours. 1-5 labeled on spectra indicate different GdnHCl concentration: 1, 0 M; 2, 0.4 M; 3, 0.6 M; 4, 1 M; 5, 2 M, respectively. Excitation was at 295 nm; emission at 310–410 nm. A: Fluorescence spectra of PP1wt; B: Fluorescence spectra of Y306K; C: Fluorescence spectra of Y306E.

by 40% (*21*). We hypothesized, therefore, that Tyr306 makes an important contribution to the behavior of PP1c. To test this hypothesis, we constructed three substitution mutants, PP1Y306A, PP1Y306K, and PP1Y306E. The effects on enzymatic activity with the peptide substrate were more pronounced than with PNPP, suggesting that PNPP is not an optimal substrate for PP1. All three substitutions increased enzyme activity with the peptide substrate, indicating that the aromatic group of Tyr reduces access of substrate to the catalytic centre of the enzyme. Tyr306 may, therefore, have a dual function, in substrate binding and in catalysis. We also measured enzymatic activity with RIIas substrate and found that the substitutions had no significant effect on substrate specificity (data not shown).

We also noted that the stability of the mutants in GdnHCl was reduced and that their fluorescence intensities fell. There are three Trp residues in PP1c (W149, W206, and W216), and the effect of the GdnHCl is presumably to perturb the active site and alter the microenvironment adjacent to the Trp residue. In 0.6 M GdnHCl, PP1Y306E and PP1Y306A lost all activity, and their emission peaks were strongly red shifted, whereas that of the wild type was unchanged. Because the conformations of all three substitution mutants differed from that of the wild type, we infer that the aromatic group of Tyr plays a crucial role in maintaining enzyme stability.

As PP1 was an important protein phosphatase regulated by both kinases and inhibitors (*22–24*), we also studied the effect of Tyr306 substitutions on inhibition by the inhibitors MCLR, TM, OA, and NCTD. We found that the sensitivity of PP1Y306A to MCLR was threefold reduced, whereas the sensitivity of PP1Y306E to TM and NCTD was reduced 15-fold and 10-fold, respectively. Because all these inhibitors except NCTD have sim-

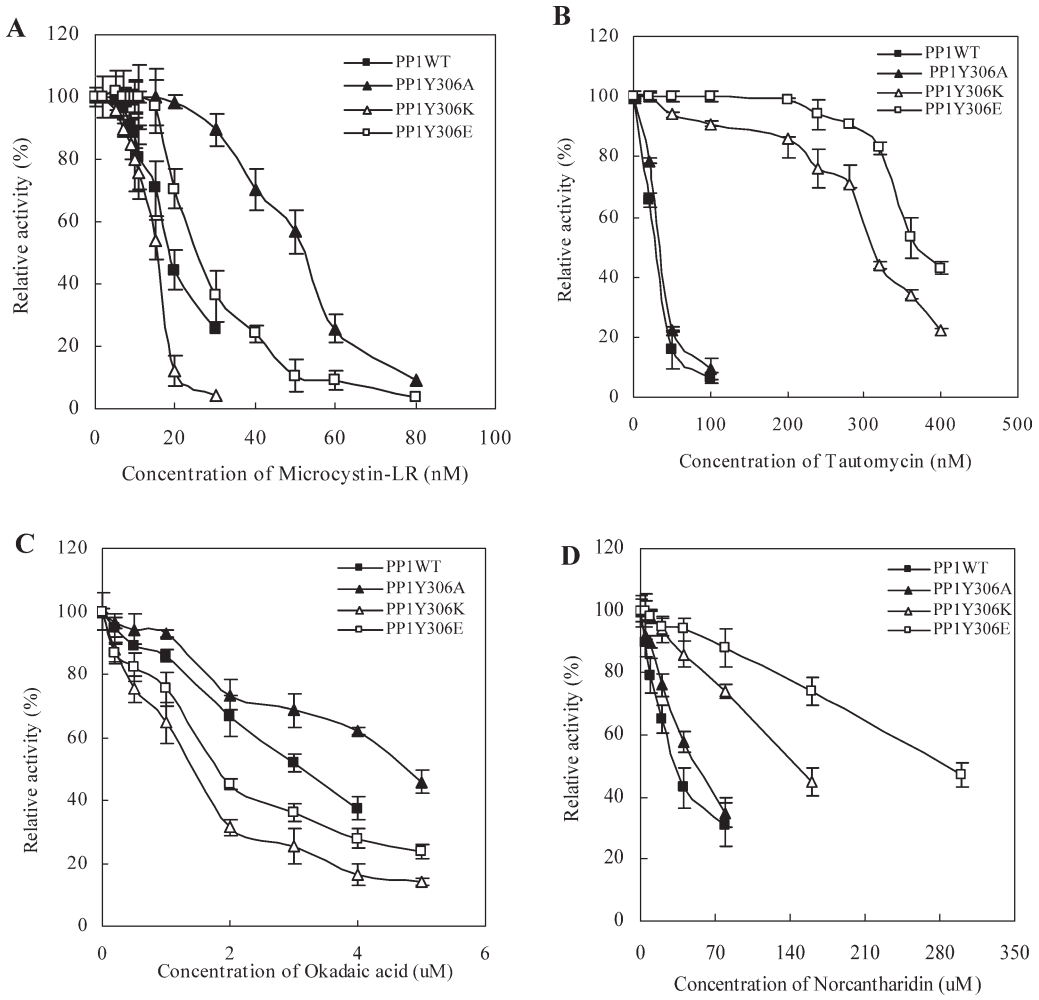

Figure 6. Inhibition of PP1wt and mutants. Purified enzymes PP1wt (■), PP1Y306A (▲), PP1Y306K (△), PP1Y306E (□) and inhibitors at different concentration were assayed using K-R-Tp-I-R-R as substrate. A: Inhibition by Microcystin-LR; B: Inhibition by Tautomycin; C: Inhibition by Okadaic acid; D: Inhibition by Norcantharidin. For details see Materials and Methods section. Each point represents the mean ± S.E.M. ($n \geq 3$).

ilar interaction sites, it seemed possible that we could identify the structural features required for toxin inhibition, and we selected the interaction of Tyr 306 with MCLR for further analysis.

The surface of PP1 has three shallow grooves radiating from the active site (*25*). These consist of a hydrophobic groove, a C-terminal groove and an acidic groove, with the active site situated at the bifurcation point of an extended Y-shaped groove. The hydrophobic groove is exposed at the surface. MCLR and TM have similar structures, with three features in common: a carboxyl group, a hydrophobic tail, and a large macrocyclic do-

Table 2
Effects of toxins and inhibitor on PP1 wild type and mutants

	IC_{50}			
Inhibitors	PP1wt	PP1Y306A	PP1Y306K	PP1Y306E
Microcystin-LR (nM)	18.0 ± 1.1	53.0 ± 2.2	13.5 ± 1.7	28.9 ± 2.2
Tautomycin (nM)	28.2 ± 1.5	35.1 ± 2.3	292.9 ± 10.5	397.8 ± 5.1
Okadaic acid (μM)	3.6 ± 0.1	6.3 ± 0.2	1.2 ± 0.1	1.7 ± 0.2
Norcantharidin (μM)	34.5 ± 2.0	49.0 ± 1.7	152.0 ± 3.3	333.0 ± 8.9

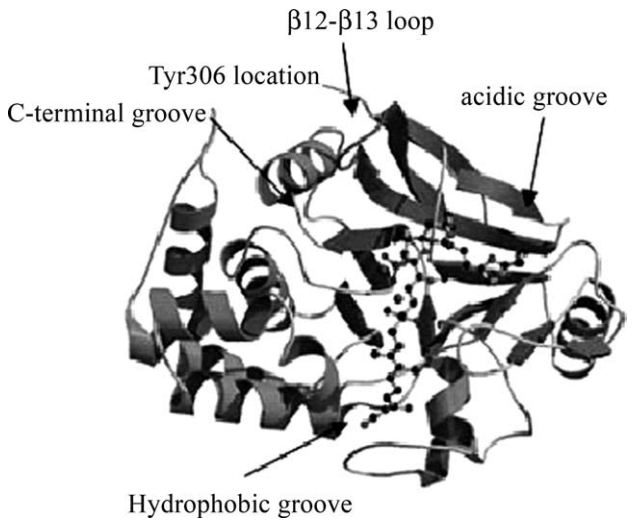

Figure 7. Proposed architecture of the PP1α-MCLR complex with the protein shown as a ribbon representation, MCLR is shown in ball-and-stick form. Tyr306 location is shown (*24*).

main (*26*). MCLR binds to PP1 in such a way as to interact with three distinct regions of the surface: the metal-binding site, the hydrophobic groove, and the edge of the C-terminal groove near the active site. Indirect coordination of MCLR to the metals occurs via two metal-liganded water molecules and involves a carboxylate group and a carbonyl oxygen of the toxin. The long hydrophobic side chain packs into the hydrophobic groove. Even though the inhibitor binds to the C-terminal groove mainly through a β12-β13 loop, Tyr 306 is located at the border of the C-terminal groove (Fig. 7). MCLR is, therefore, likely to bind to several amino acids in the C-terminal groove, so inhibiting enzyme activity and suggesting that Tyr306 may be a site of interaction with the inhibitor. So we deduce that Tyr306 is very possibly the major interaction site for MCLR. TM maybe interact with PP1 in similar way.

NCTD, a demethylated form of cantharidin, is also a strong inhibitor of PP1 and PP2A. Both inhibitors have low molecular weights (168 Da), whereas the molecular weights of other toxins such as OA, microcystins, and calyculin A are in the range 500–1,000 Da. So NCTD maybe interact with enzymatic molecule in different way than other inhibitors. It was reported that the anhydride rings of NCTD are hydrolyzed and it binds to the catalytic sites of Ser/Thr phosphatases PP5 in a unique conformation (*27*). The catalytic domain of PP5 (PP5c) shares 35–45% sequence identity with the catalytic domains of the other members of the PPP family. Hence, we thought NCTD inhibition to PP1 might interact with active centre of PP1 in a special interaction conformation like PP5, and Tyr306 participated in this process. So we concluded that Tyr306 maybe also interaction site of these inhibitors.

In conclusion, substitutions of Tyr306 led to increased substrate affinity and a reduction in enzyme stability. This site may also be the site of interaction of several inhibitors, including MCLR, TM, and NCTD. All our findings suggest that Tyr 306 plays an important role in maintaining the stability of PP1 and affecting its sensitivity to inhibitors.

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