The nonconserved N-terminus of protein phosphatases 1 influences its active site

XiuJie. Xie[1,2], Wei Huang[2], ChengZhe. Xue[2], Qun Wei[2,]*

[1]Tianjin Key Laboratory of Environmental Remediation and Pollution Control/College of Environmental Science and Engineering, Nankai University, Tianjin, 300071, P. R. China [2]Department of Biochemistry and Molecular Biology, Beijing Normal University, Beijing Key Lab, Beijing, 100875, P.R. China

Protein phosphatase 1 consists of a secondary structure arrangement, conserved in the serine/threonine protein phosphatase gene family, flanked by nonconserved N-terminal and C-terminal domains. The deletion mutant of PP1 with the 8 nonconserved N-terminal residues removed was designated PP1-(9-330). PP1-(9-330) had a higher activity and affinity than PP1 when assayed against four different substrates, and it also demonstrated a 6-fold higher sensitivity to the inhibitor okadaic acid. This suggested that the N-terminal domain suppresed the activity of PP1 and interfered with its inhibition by okadaic acid. The ANS fluorescence intensity of PP1-(9-330) was greater than that of PP1, which implies that the hydrophobic groove running from active site in the truncated PP1 was more hydrophobic than in PP1. Our findings provide evidence that the nonconserved N-terminus of PP1 functions as an important regulatory domain that influences the active site and its pertinent properties. [BMB reports 2008; 41(12): 881-885]

INTRODUCTION

PP1 is a member of serine/threonine protein phosphatase gene family (PPP gene family) that controls multiple cellular functions and plays crucial roles in cellular signal transduction. The catalytic subunit of PP1 (PP1C) interacts with different regulatory and targeting subunits to form distinct PP1 holoenzymes that regulate different cellular processes (1-5). The x-ray crystal structure of the PP1 catalytic subunit shows that there are three grooves (the hydrophobic, C-terminal, and acidic grooves) on the surface of PP1. The active site of PP1 lies at the confluence of the three grooves. PP1 is a metalloprotein that possesses a bimetal center at the active site, which is a potential binding site for substrates and inhibitors (6-8). The catalytic subunits of PPP family members have con-

served catalytic cores, which share very high sequence similarity, an identical secondary structure arrangement, as well as a homologous three-dimensional structure. Sequence alignment and secondary structure arrangements had been presented for PPP family members (9). The x-crystal structure shows that PP1 conserved catalytic core possess 10 α helices and 14 β strands, which is flanked by nonconserved N-terminal and C-terminal domains (Fig. 1) (6, 10).

Among the nonconserved domains, the C-terminus and β12-β13 loop of PP1 have been investigated extensively (11-15). However, except for a study showing that deletion of 4 or 19 N-terminal amino acids of PP1c results in loss of expression, few experiments have been performed on the non-conserved N-terminus of PP1c (16).

The PP1c N-terminus, up to the first amino acid of the first conserved domain, consists of the 8 amino acids MSDSEKLN (Fig. 1). In order to explore the roles of this nonconserved
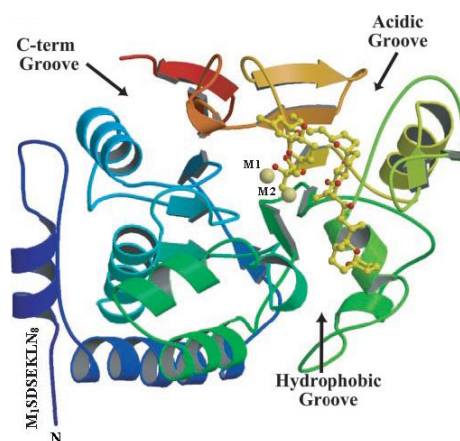
Fig. 1. Architecture of the PP1-OA complex. The protein is shown as a ribbon diagram, the coloring is *blue* at the N terminus and *red* at the carboxyl terminus. The nonconserved N-terminus amino acids residues are shown. The conserved core is composed of 10α helices and 14β strands. The hydrophobic groove, C-terminal groove, and acidic groove converge at the active site, which contains two metal ions (designated M1 and M2). OA is shown in a ball-and-stick form with carbon atoms in yellow and oxygen atoms in red. (reproduced from Fig. 1A of reference 6).

N-termini in PP1 function, we constructed a deletion mutant, PP1-(9-330), which removes the amino acids 1-8 from the N-terminus. We have compared the properties of PP1 and PP1-(9-330).

RESULTS

Preparation of proteins

PP1-(9-330) was purified by the same method as PP1, although a subtle distinction was noted in the elution conditions. PP1-(9-330) required a higher salt concentration for elution from heparin-agarose than PP1. This suggested that the deletion of the N-termini affected the affinity of the phosphatase for heparin, which is a type of PP1 inhibitor. The yields of PP1 and PP1-(9-330) were comparable at about 10 mg per 1 liter of culture. PP1 and PP1-(9-330) were purified to electrophoretic purity.

Phosphatase assays

PP1 and PP1-(9-330) were assayed using ^{32}P-labeled Pho.a, ^{32}P-labeled MBP, ^{32}P-labeled RII, as well as PNPP as substrates. Except for the low specific activity of PP1 with the RII peptide, all the specific activities of PP1 and PP1-(9-330) for these substrates were high. Even though the RII peptide is a poor substrate of PP1, it was an excellent substrate for the truncated PP1. The specific activities of PP1-(9-330) were 2-fold, 2.6-fold, 23.8-fold, and 1.33-fold higher than those of PP1 with these substrates, respectively. Fig. 2 shows the higher activities of PP1-(9-330) relative to PP1. The kinetic parameters of PP1-(9-330) and PP1 are shown in Table 1. The *Kms* of

PP1-(9-330) were lower than those of PP1 and the *Vmaxs* were greater than those of PP1 with ^{32}P-labeled Pho.a, ^{32}P-labeled MBP, ^{32}P-labeled RII, as well as the PNPP substrate.

Okadiac acid sensitivity of phosphatases

The sensitivity of PP1-(9-330) and PP1 to OA were compared. Fig. 3A shows that OA inhibited PP1-(9-330) with a 6-fold lower IC_{50} (5 nM) than PP1 (30 nM). The IC_{50} value of PP1 in this study was consistent with those reported by previous researchers (17). These observations suggested that PP1-(9-330) was more sensitive to OA than wild-type PP1, which retained its 8 N-terminal amino acids. In other words, the sensitivity of this phosphatase to OA increased following deletion of its N-terminus region.

Table 1. Kinetic parameters of PP1 and PP1-(9-330)

	Substrates							
	Pho.a		MBP		RII		PNPP	
	Km	Vmax	Km	Vmax	Km	Vmax	Km	Vmax
PP1	4	17000	50	33333	96	1667	10	10000
PP1-(9-330)	1.9	33333	20	67000	6.3	30000	8	13333

^{32}P-labeled Pho.a, ^{32}P-labeled MBP, ^{32}P-labeled RII peptide and PNPP were used as substrates, and the K_m values for ^{32}P-labeled substrates are expressed in micromoles, while those for PNPP are in millimoles. All V_{max} values are in nanomoles of Pi released per minute per milligram of protein.
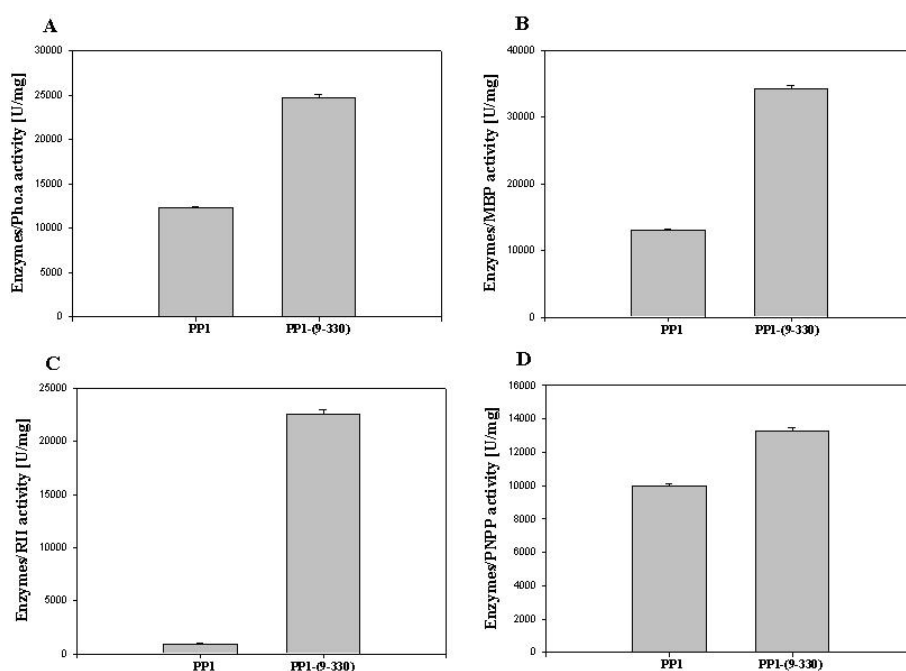

Fig. 2. The specific activities of PP1 and PP1-(9-330). The specific activities of PP1 and PP1-(9-330) are shown with (A) ^{32}P-labeled Pho.a, (B) ^{32}P-labeled MBP, (C) ^{32}P-labeled RII peptide, or (D) PNPP as substrate. All data are the mean ± S.D. of four experiments.
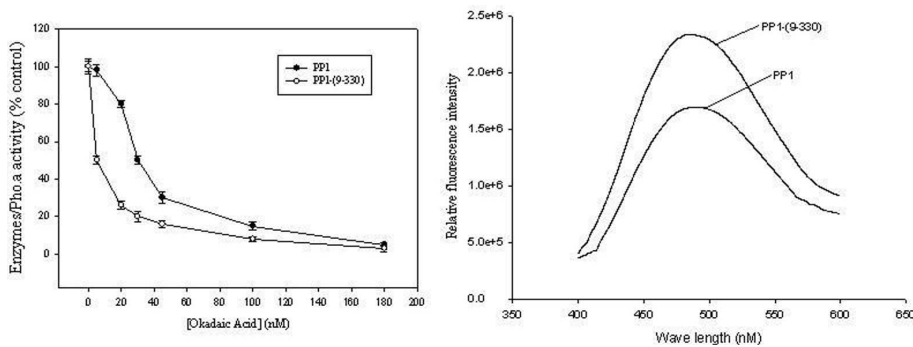
ANS fluorescence spectroscopy

The exposure of the hydrophobic groove of PP1 and PP1-(9-330) was studied by measuring changes of the fluorescence spectra using the hydrophobic probe ANS. The ANS fluorescence intensity of PP1-(9-330) was greater than that of PP1, as shown in Fig. 3B, and the emission wavelength maximum was blue-shifted from PP1 to PP1-(9-330). These observations imply that the groove in the truncated PP1 was more hydrophobic than in PP1.

DISCUSSION

The nonconserved N-terminus of PP1c consists of 8 amino acid residues. PP1-(9-330), which has these 8 amino acids deleted, has higher *Vmax* and lower *Km* values with ^{32}P-labeled Pho.a, ^{32}P-labeled MBP, ^{32}P-labeled RII peptide, and PNPP substrates compared with PP1. The kinetic parameters reveal that PP1 has the lowest *Km* (or the highest *Vmax*) with Pho.a and the highest *Km* (or the lowest *Vmax*) with RII, which suggest that the affinity (or the catalytic efficiency) of PP1 is the highest for Pho.a and the lowest for RII. The *Kms* (or the *Vmaxs*) of PP1 for MBP and PNPP are in the middle, therefore the affinities (or the catalytic efficiency) of PP1 for MBP and PNPP are in the middle. This result is consistent with the previous conclusion that Pho.a is a specific substrate of PP1 while MBP is a substrate shared by other Ser/Thr protein phosphatases and is also an excellent substrate for PP1, whereas the RII peptide is a poor substrate for PP1. The *Kms* (or the *Vmaxs*) of PP1-(9-330) have the same trend as PP1 in regard to these substrates. When compared with PP1, PP1-(9-330) has an increased substrate affinity (or catalytic efficiency) due to the decreased *Km* (or increased *Vmax*) for each substrate, especially for RII. The change in specific activity is similar to that of *Vmax* in PP1 and PP1-(9-330). All these results suggest that the nonconserved N-terminus of PP1 weakens the substrate affinity and catalytic efficiency of the enzyme.

PP1-(9-330) is 6-fold more sensitive to OA than PP1, which implies that the nonconserved N-terminus may oppose inhibition by OA. The crystal structure of OA bound to PP1 shows that OA binds and contacts residues in the hydrophobic groove. The hydrophobic groove runs from the active site and is exposed at the surface of PP1 where it can be recognized and bound by a large number of molecules, including both substrates and inhibitors (6, 7). The hydrophobic groove is far from the N-terminus of PP1; therefore, it would seem that the N-terminus of PP1 should not affect this hydrophobic groove, but this is not the case. The crystal structure of OA bound to PP1 (6), shown here as Fig. 1, shows that a cluster of 7 α helices is located between the hydrophobic groove and the nonconserved N-terminus. Deletion of the N-terminus may disturb interactions between the N-terminal sequence and residues in these helices and, therefore, influence the position and orientation of each helix within the clusters and successively affect the active site and surface hydrophobicity of the hydrophobic groove. A change in the active site and surface hydrophobicity of the hydrophobic groove in PP1-(9-330), as a result of the N-terminal deletion, may alter its sensitivity to OA.

To further study the interrelation between the hydrophobic groove and the nonconserved N-termini, we measured the ANS fluorescence spectra of PP1-(9-330) and PP1, with the results presented in Fig. 3B. The ANS fluorescence intensity of PP1-(9-330) was higher than that of PP1 and its emission peak was blue-shifted relative to PP1, which confirmed that the extreme N-terminus of PP1 influences the hydrophobicity of the hydrophobic groove and active site and thus modulates enzymatic activity and sensitivity to OA. Deletion of the N-terminus could result in an increase in the surface hydrophobicity of the hydrophobic groove in PP1-(9-330), and the altered hydrophobic groove and active site maybe aquire a more open position, facilitating the access of substrates and inhibitors. OA was selected as an inhibitor in these experiments because hydrophobic interactions between it and PP1 predominate. This observation may help to explain the remarkable selectivity for distinct PP1 isoforms displayed by PP1 regulators.

Further studies are clearly needed to investigate the roles of the nonconserved N termini in the serine/threonine protein phosphatase gene family. An increased understanding should pave the way for the design of novel protein Ser/Thr phosphatase inhibitors and activators for use as therapeutic agents.

MATERIALS AND METHODS

Materials

The PP1 plasmid and pTACTAC expression vector were kindly provided by Dr. P. T. W. Cohen of Dundee University. The E. coli strain DH5 α was from our laboratory. OA and PNPP were purchased from Sigma Chemical Corp., RII peptide from BioMol Research Labs Inc., the catalytic subunit of PKA from Promega Chemical Corp and $(\gamma\text{-}^{32}p)$ATP from Beijing Furi Biology Engineering Corp. $PO(OC_6H_5)=NCH=CC_6H_5)$ and $POPOP\{(OC(C_6H_5)=CHN=C)_2C_6H_4\}$ were obtained from E. Merck (Dowex, England), and MBP was kindly provided by Zhixing Wang of Tshinghua University. All other chemicals were of standard laboratory grade, unless otherwise stated.

Construction of vectors

The deletion mutant of PP1, PP1-(9-330), was constructed by PCR. The primers for PP1-(9-330) were; 5′primer, 5′-TAT ACA TAT GCT GGA CTC TAT CAT CGG GC3′ and 3′ primer, 5′-GGC GCG TAC TAT GGT TGC TTT GAC 3′. An NdeI site with an initiating Met codon was inserted into the 5′ primer sequence. The PCR product of PP1-(9-330) was digested with *Nde*I and *Hind*III, ligated into pTACTAC, and then transformed into DH5α.

Expression and purification of proteins

For expression and purification of PP1-(9-330) we used the method previously described for PP1 (18). Enzyme purity was assessed by 12% SDS-PAGE and protein concentration was measured using the Bradford method.

Preparation of ^{32}P phosphorylated substrates

^{32}P-labeled Pho.a was prepared by the procedure of Cohen (19), and phosphorylase b and phosphorylase kinase were isolated from rabbit muscle using published methods (18, 20). The RII peptide was labeled with ^{32}P as previously described (21). MBP was phosphorylated as previously described (22), with slight modifications. In brief, MBP (1.83 mg) was mixed with PKA (80 units) and 5 mM $(\gamma\text{-}^{32}p)$ ATP (200 mci/mmol) in 50 mM Tris-HCl (pH 7.4), 10 mM magnesium chloride, 1 mM EGTA, 2 mM DTT, and 0.01% Brij 35, and then incubated for 6 hours at 30°. Residual ATP was then removed by dialysis.

Assay of protein phosphatases

Enzymes were assayed using ^{32}P-labeled Pho.a as a substrate, as previously described (19). The concentration of ^{32}P-labeled Pho.a was 10 μM for activity assays and 2.5 μM, 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, or 160 μM for kinetic assays in a final 30 μl assay mixture. With ^{32}P-labeled MBP as a substrate, assays for activity and kinetics were performed according to the instructions provide with the protein serine/threonine phosphatase (PSP) assay system (22). The concentration of ^{32}P-labeled MBP was 25 μM for activity assays and 6.25 μM, 12.5 μM, 25 μM, 50 μM, 100 μM, or 200 μM for kinetic assays. For RII, activity was determined by measuring the release of ^{32}p from the RII peptide. The enzyme solution (10 μl) was mixed with 10 μl assay buffer (40 μM ^{32}P-labeled RII peptide, 75 mM Tris-HCL [pH7.4], 2 mM MnCL$_2$, 2 mM DTT, 0.15 mM Na$_2$EDTA, and 0.015% Brij-35) at 30° for 10 min. The concentration of ^{32}P-labeled RII was 5 μM, 10 μM, 20 μM, 40 μM, 80 μM, or 160 μM for kinetic assays. Assays for activity and kinetics with PNPP were carried out as previously described (23). ^{32}P release was restricted to 15-20% of the total counts present in the assays (24). One unit of phosphatase activity was defined as one nmol of PNPP hydrolyzed or ^{32}Pi released from the labeled substrate per milligram of enzyme per min at 30°C.

Inhibition and activation of phosphatases

With ^{32}P-labeled Pho.a as a substrate, the inhibition assays with OA were performed as previously described (25).

Measurement of ANS fluorescence

The ANS florescence spectrum was recorded on a FLORMAX-2 fluorometer. PP1 and PP1-(9-330) were diluted to 5 μM. 1‰ (v/v) ANS (50 mM) was added, the mixtures were incubated for 2-4 hours at 4°C, and the fluorescence spectra were recorded at room temperature in a 1 cm path length cuvette with an excitation wavelength of 345 nm and a slit of 10 nm. Emission was measured from 400 nm to 600 nm.

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