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

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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 conserved 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
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 32P-labeled Pho.a, 32P-labeled MBP, 32P-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 Km's of PP1-(9-330) were lower than those of PP1 and the Vmax's were greater than those of PP1 with 32P-labeled Pho.a, 32P-labeled MBP, 32P-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 IC50 (5 nM) than PP1 (30 nM). The IC50 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)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Pho.a</th>
<th>MBP</th>
<th>RII</th>
<th>PNPP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>Vmax</td>
<td>Km</td>
<td>Vmax</td>
</tr>
<tr>
<td>PP1</td>
<td>4</td>
<td>17000</td>
<td>50</td>
<td>33333</td>
</tr>
<tr>
<td>PP1-(9-330)</td>
<td>1.9</td>
<td>33333</td>
<td>20</td>
<td>67000</td>
</tr>
</tbody>
</table>

32P-labeled Pho.a, 32P-labeled MBP, 32P-labeled RII peptide and PNPP were used as substrates, and the Km's for 32P-labeled substrates are expressed in micromoles, while those for PNPP are in millimoles. All Vmax values are in nanomoles of Pi released per minute per milligram of protein.
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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 32P-labeled Pho.a, 32P-labeled MBP, 32P-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 Vmaxes) 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 Vmaxes) 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 acquire 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 isomers 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 DH5α 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 (γ-32p)ATP from Beijing Furi Biology Engineering Corp. PO(OCC6H5)=NCH=CC6H5 and POPOP{OC(C6H5)=CHN=C6H5} 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 CCT GGA CTC TAT GCT GGA CTC TAT CAT CGG GC3' and 3' primer, 5'-GCC GCC TAC TAT GGT TGC TTT GAC 3'. An Ndel site with an initiating Met codon was inserted into the 5' primer sequence. The PCR product of PP1-(9-330) was digested with Ndel and HindIII, 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 32P phosphorylated substrates
32P-labeled Pho.a was prepared by the preparation 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 32P 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 (γ-32p) 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 32P-labeled Pho.a as a substrate, as previously described (19). The concentration of 32P-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 32P-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 32P-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 32p from the RII peptide. The enzyme solution (10 μl) was mixed with 10 μl assay buffer (40 μM 32P-labeled RII peptide, 75 mM Tris-HCl [pH7.4], 2 mM MnCl2, 2 mM DTT, 0.15 mM Na2EDTA, and 0.015% Brij-35) at 30° for 10 min. The concentration of 32P-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). 32P 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 32Pi released from the labeled substrate per milligram of enzyme per min at 30°C.

Inhibition and activation of phosphatases
With 32P-labeled Pho.a as a substrate, the inhibition assays with OA were performed as previously described (25).

Measurement of ANS fluorescence
The ANS fluorescence 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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