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Regulation of the catalytic domain of protein phosphatase 1 by the terminal region of protein phosphatase 2B

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C-terminal regions of the protein phosphatases PP1 and PP2B were seldom studied. C-terminal 24 amino acids of PP1 was deleted, its enzymatic activity increased 3-fold while its stability declined. When the truncated PP1 was fused with the terminal (residues 483–511) of PP2B, both its enzymatic activity and its stability remained low. This indicates that the termini of PP2B and PP1 have inhibitory effect on the catalytic domain of PP1. PP1-(1–306) and PP1wt differ in their activation by metal ions, showing that the sites interacting with metal ions are not located in its C-terminus; while metal ions activated notably to PP1/PP2B chimera. In addition, the sensitivity results of PP1-(1–306) to the inhibitors, TM and NCTD, proved that these two inhibitors also did not bind to the C-terminus. However, the IC_{50}s of PP1/PP2B chimera were higher than for PP1-(1–306), indicating that the C-terminal region interferes interactions with these inhibitors to some extent. Although 483–511 segment of PP2B was not the functional domain, it played important role in interaction with metal ions and inhibitors. It further indicates although PP1 and PP2B have high sequence identity, their non-conserved termini have different roles.

Keywords: PP1/PP2B/C-terminus/chimera/stability/metal ions/inhibitor.

Abbreviations: ANS, 1-anilinonaphthalene-8-sulphonic acid; CAN, calcineurin A subunit; DARPP-32, dopamine and cAMP-regulated phosphoprotein Mr 32,000; GdnHCl, guanidine hydrochloride; MCLR, microcystin-LR; NCTD, norcantharidin; OA, okadaic acid; PNPP, *p*-Nitrophenyl Phosphate; PP1, type-1 serine/threonine protein phosphatase; PP2B, type-2B serine/threonine protein phosphatase; TM, tautomycin.

Protein phosphatase-1(PP1) and protein phosphatase 2B (PP2B, calcineurin) are major serine/threonine protein phosphatases that are involved in many biological processes, including immune responses, signalling via the cAMP pathway, glycogen metabolism, muscle contraction and mitosis (*1–3*). PP2B is also subject to inhibition by the heat-stable inhibitors of PP1 (inhibitor 1, DARPP-32). In addition to directly regulating cellular activities, PP1 and PP2B also indirectly regulate enzymatic activity by interacting with each other. For example, in neurons, PP2B can dephosphorylate the inhibitors DARPP-32 and I-1 and so activate PP1 (*4–6*).

PP2B is a heterodimer composed of a catalytic subunit A and a regulatory subunit B. The catalytic subunit of PP1 has high sequence identity with the catalytic subunits of PP2B, but differs in their substrate specificities and the regulations by different agents. Their catalytic domains have 40% sequence identity, whereas their N- and C-terminal regions are very different (*7*). The catalytic subunit of PP2B (CNA), unlike that of PP1 (PP1c), has three regulatory domains in its C-terminal region: a CNB-binding domain (BBH), CaM-binding domain (CBD) and autoinhibitory domain (AID) (*8–10*).

Our previous work using CaM-fusion methodology showed that the catalytic domain of PP2B is regulated by binding of different segments of its catalytic subunit with a number of C-terminal regions, such as Ci511 (389–511), Ci482 (389–482), Ci456 (389–456), Ci413 (389–413) and the AID peptide (457–482). The extent of inhibition conferred by the AI domain was depended on the length of the segment examined. The inhibitory activity of the complete C-terminus (389–511, i.e. from the CBD to the terminus) was the strongest, followed by the region of 389–482 (which only contains the CBD and AID regions). We also showed that the C-terminal segment (483–511) of PP2B, which has seldom been examined because it lacks any notable functional domains, also contributed to autoinhibition (*11*). The inhibitory activity of AID was the weakest.

About C-terminal of PP1, Zhang *et al*. (*12*) reported that deletion of 33 and 25 amino acids residues at the C-terminus yielded-specific activities of 9 and 61% of wild-type (wt), respectively. All deletions of the C-terminus beyond residue 33 led to complete loss of activity. The active centre structure of PP1 is similar to that of PP2B (*13*, *14*). In this work to be presented here we find that deletion of the PP1 C-terminus leads to an increase in enzymatic activity while the stability of the enzyme decline. In order to see whether the interaction of the catalytic domain of PP1 with its C-terminal region is similar to the corresponding interaction in PP2B, we connect PP1-(1–306) with the C-terminus (residues 483–511) of PP2B. We find that the C-terminus of PP2B, like that of PP1 itself, has an inhibitory effect on the enzyme; but it cannot make

molecular structure compact. Examination of the activation of PP1 by metal ions shows that PP1-(1–306) has properties similar to those of CNAa (the catalytic domain of PP2B), but its sensitivity to the inhibitors TM and NCTD is similar to those of PP1wt. Moreover, observations of the effects of metal ions and inhibitors show that although the 483–511 segment of PP2B lacks any obvious functional domains, it plays an important role in the interactions of PP2B with metal ions and inhibitors. These results indicate that these non-conserved domains of same family protein phosphatases play different roles in biological function, and it provides insight into the possible architecture of other less well studied protein phosphatases.

Materials and Methods

Materials

The PP1 plasmid and pTACTAC expression vector were kindly provided by Dr P.T.W. Cohen of Dundee University. Strains DH5α and BL-21, and the PP2B plasmid, were from our own laboratory. 1-anilinonaphthalene-8-sulphonic acid (ANS), Norcantharidin (NCTD), Okadaic acid (OA), Microcystin-LR (MCLR) and PNPP were purchased from Sigma Chemical Corp. The substrate (K-R-Tp-I-R-R) was synthesized by Scilight Biotechnology, LLC (China). Malachite green and Tautomycin (TM) were purchased from BIOMOL International, LP. Other chemicals were of standard laboratory grade, unless otherwise stated. The PP1 mutants were all based on rabbit muscle PP1α isoform cDNA.

Construction of chimeric mutants of PP1 and PP2B in pTACTAC vector

We made a truncated mutant of PP1 and a chimeric construct. The chimeric mutant contained PP1-(1–306) and the PP2B C-terminal region; it was constructed by splicing by over-lap extension PCR (SOE), and inserted into pTACTAC vector. The 5′primer for PP1/PP2B Ch: 5′ TATA CAT ATG TCC GAC AGC GAG AAG CTC 3′ (NdeIsite underlined). The linking 3′ primer was: 5′: GGC AAG TAC GAC GCC AAC CTT AAC TCC ATC3′, and the segment used with PP1 catalytic subunit wt plasmid as template. The linking 5′primer was: 5′: GTT GGC GTC GTA CTT GCC CTT GTT CTT GTC3′; 3′primer: 5′GC AAG CTT TCA CTG AAT ATT GCT 3′ (Hind III cutting sites was underlined). The segment used with PP2B catalytic subunit wt plasmid as template. Construction of PP1 (1–306) referenced the paper (*15*). The constructs were then used to transform competent *Escherichia coli* DH5αcells.

Expression and purification of PP1wt and mutants

Expression and purification were performed as for PP1 (*16*). Enzyme purity was assessed by 12% SDS–PAGE, and protein concentrations were measured by the method of Bradford.

Protein phosphatase activity

Assays were carried out by mixing 25 µl Ser/Thr assay buffer (20 mM MOPS, pH 7.5, 1 mM DTT, 0.15 MNaCl, 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, and incubating further at 30°C for 30 min. Malachite green solution of 100 µl was then added to each reaction, and after incubation for 15 min, phosphate release was quantified by measuring absorbance at 630 nm with a microtitre plate reader. All reactions were performed in triplicate. One unit of phosphatase activity was defined as the amount of enzyme required to hydrolyse 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 as described (*16*).

Fluorescence spectroscopy

Fluorescence measurements were performed using a FLORMAX-2 fluorometer. Enzymes (5 µM) in buffers containing various concentrations of GdnHCl (0 M; 0.4 M; 1 M) were allowed to reach chemical and thermal equilibrium at 4°C for 24 h. They were then excited at 295 nm using 5 nm excitation and emission slit widths, and their emission spectra were recorded between 310 nm and 410 nm to measure intrinsic fluorescence. Their extrinsic fluorescence was also recorded as follows: the enzymes (5 µM) were treated as for intrinsic fluorescence, and 50 µM ANS was then added; the mixtures were incubated for 2–4 h at 4°C and their fluorescence spectra were recorded at room temperature in a 1-cm path length cuvette with an excitation wavelength of 345 nm. Emission spectra were measured from 400 nm to 600 nm.

Protein phosphatase inhibition assays

Inhibition was assayed with K-R-Tp-I-R-R as substrate. Inhibitors (Microcystin-LR, Tautomycin, Okadaic acid, Norcantharidin) were incubated with enzyme for 5 min at 30°C prior to addition of substrate. Each determination was performed in triplicate and IC_{50} values were calculated.

Effect of metal ions on protein phosphatase activity

PP1wt and mutants were treated with 5 mM EDTA pH 7.0, for 2 h at 4°C, and extensively dialysed against 50 mM MOPS, pH 7.0, 500 mM KCl, 0.2 mM EDTA and 50% glycerol, in order to remove Mn^{2+}. Various concentrations of metal ions were incubated with the substrate for 10 min at 30°C and reactions were initiated by addition of enzyme. Activity was measured as above.

Results

Enzymatic activity and kinetic parameters

We constructed the C-terminal deletion mutant of PP1 [PP1(1–306)] and the chimeric mutant with PP2B (PP1/PP2B Ch). Fig. 1 is a schematic representation of PP1, PP2B and PP1/PP2B Ch. The mutants [PP1(1–306) and PP1/PP2B Ch] and wt PP1 were purified to near-homogeneity (Fig. 2).

The specific activities of the phophatases were determined with PNPP and K-R-Tp-I-R-R as substrates. The specific activities of PP1(1–306) (*14*) and PP1/PP2B Ch with PNPP as substrate were $741.69*10^{-3} \pm 0.01$ U/mmol and $530.81*10^{-3} \pm 0.01$ U/mmol, respectively, both notably higher than that

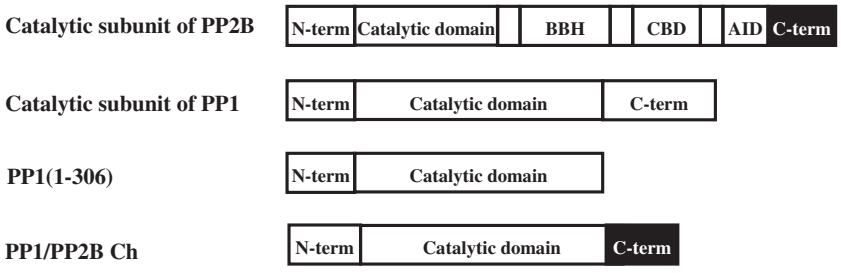

Fig. 1 Schematic representation of the catalytic subunits of PP2B, PP1 and their mutants.

of PP1wt ($283.16*10^{-3}$ U/mmol). We also found that the activity of PP1 (1–306) with K-R-Tp-I-R-R as substrate was $96.09*10^{-3}$ U/mmol, 3.5-fold higher than that of wt ($25.41*10^{-3}$ U/mmol), while the activity of PP1/PP2B Ch ($18.46*10^{-3}$ U/mmol) was obviously lower than that of PP1 (1–306).

The kinetic constants were also measured with PNPP and K-R-Tp-I-R-R as substrates (Table 1). The K_{cat}/K_m of PP1(1–306) ($10{,}116 \pm 35.51$/Mol/l/s) and that of PP1/PP2B Ch ($5{,}866 \pm 21.13$/Mol/l/s) with K-R-Tp-I-R-R as substrate were 16- and 9-fold higher than that of the wt (623 ± 14.62/Mol/l/s), and their K_{cat} values (3.83 ± 0.23/s; 0.69 ± 0.14/s) were 16- and 3-fold higher, respectively. The V_{max} of PP1 (1–306) with PNPP as substrate ($62{,}500 \pm 343.45$ μmol/l/min), was also 5-fold higher than that of PP1wt ($12{,}000 \pm 148.62$ μmol/l/min), while the V_{max} of PP1/PP2B Ch ($24{,}295 \pm 234.43$ μmol/l/min) was lower than that of PP1 (1–306).

Stability of PP1wt and mutants

The stability of PP1/PP2B Ch in the denaturant guanidine hydrochloride (GdnHCl) was obviously lower than that of PP1wt (Fig. 3), and PP1 (1–306) showed a similar tendency. In 0.2 M GdnHCl the activities of PP1 (1–306) and PP1/PP2B Ch fell by about 55%, while that of PP1wt showed no obvious decrease. In 0.6 M GdnHCl, the two derivatives lost all activity, while the wt retained 80% activity.

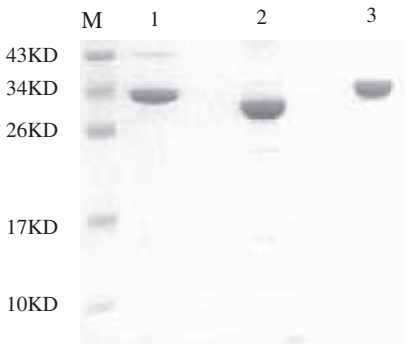

Fig. 2 SDS–PAGE electrophoresis of PP1 and its derivatives. Lane 1: PP1wt; Lane2: PP1(1–306); Lane3: PP1/PP2B Ch. Markers are 43KD, 34KD, 26KD, 17KD and 10KD.

Structural bases of the low stability in denaturant

In order to determine whether absence of the C-terminus and/or fusion with the C-terminus of PP2B affected the conformation of PP1, we examined the effect of GdnHCl on the fluorescence spectra of PP1wt and the derivatives (Fig. 4). From the peak of the intrinsic fluorescence spectrum of PP1wt (Fig. 4A) did not change significantly in 0.4 M GdnHCl; in 1 M GdnHCl it was red-shifted 3 nm. On the other hand, the mission peak of PP1 (1–306) was blue-shifted ~10 nm with respect to that of the wt in the absence of denaturant. Its fluorescence intensity markedly decreased in 0.4 M GdnHCl and the emission peak was red-shifted by 4 nm. In 1 M GdnHCl the emission peak was further red-shifted by 4 nm (Fig. 4C). The fluorescence changes of PP1/PP2B Ch were different from those of PP1 (1–306) (Fig. 4E), fluorescence intensity of PP1 (1–306) descended more notably than that of PP1/PP2B Ch. Its emission peak of PP1/PP2B Ch was red-shifted 2 and 5 nm, respectively, in 0.4 M and 1 M GdnHCl. Measurements of extrinsic fluorescence also showed that the changes in the mutants were different from those of PP1wt (Fig. 4B, D and F). They had similar rules with changes of intrinsic fluorescence.

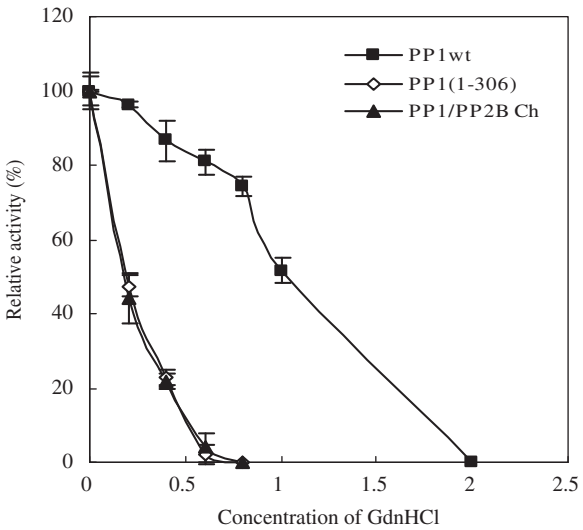

Fig. 3 Sensitivity of PP1wt and derivatives to GdnHCl denaturation. Purified PP1wt (filled square), PP1(1–306) (open diamond) and PP1/PP2B Ch (filled triangle) are assayed using PNPP as substrate. Enzymes (5 μM) are incubated with different concentration of GdnHCl at 4°C for 24 h.

Table I. Kinetic analysis of PP1wt and mutants[a].

	K-R-Tp-I-R-R			PNPP		
Enzyme	K_{cat}/K_m	K_m	K_{cat}	K_{cat}/K_m	K_m	V_{max}
PP1wt	623 ± 14.62	385 ± 19.42	0.24 ± 0.13	11.3 ± 0.36	11.0 ± 1.34	$12{,}000 \pm 148.62$
PP1(1–306)	$10{,}116 \pm 35.51$	378 ± 12.34	3.83 ± 0.23	10.8 ± 0.78	18.9 ± 2.46	$62{,}500 \pm 343.45$
PP1/PP2B Ch	$5{,}866 \pm 21.13$	117 ± 8.62	0.69 ± 0.14	3.4 ± 0.24	10.6 ± 1.61	$24{,}295 \pm 234.43$

[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 per second. Values for K_{cat}/K_m are in Molar per litre per second. V_{max} values are in micomolar per litre minure. K_m values for PNPP are in millimolar. Values for K_{cat}/K_m are in Molar per litre per second. V_{max} values are in millimolar per litre per minute

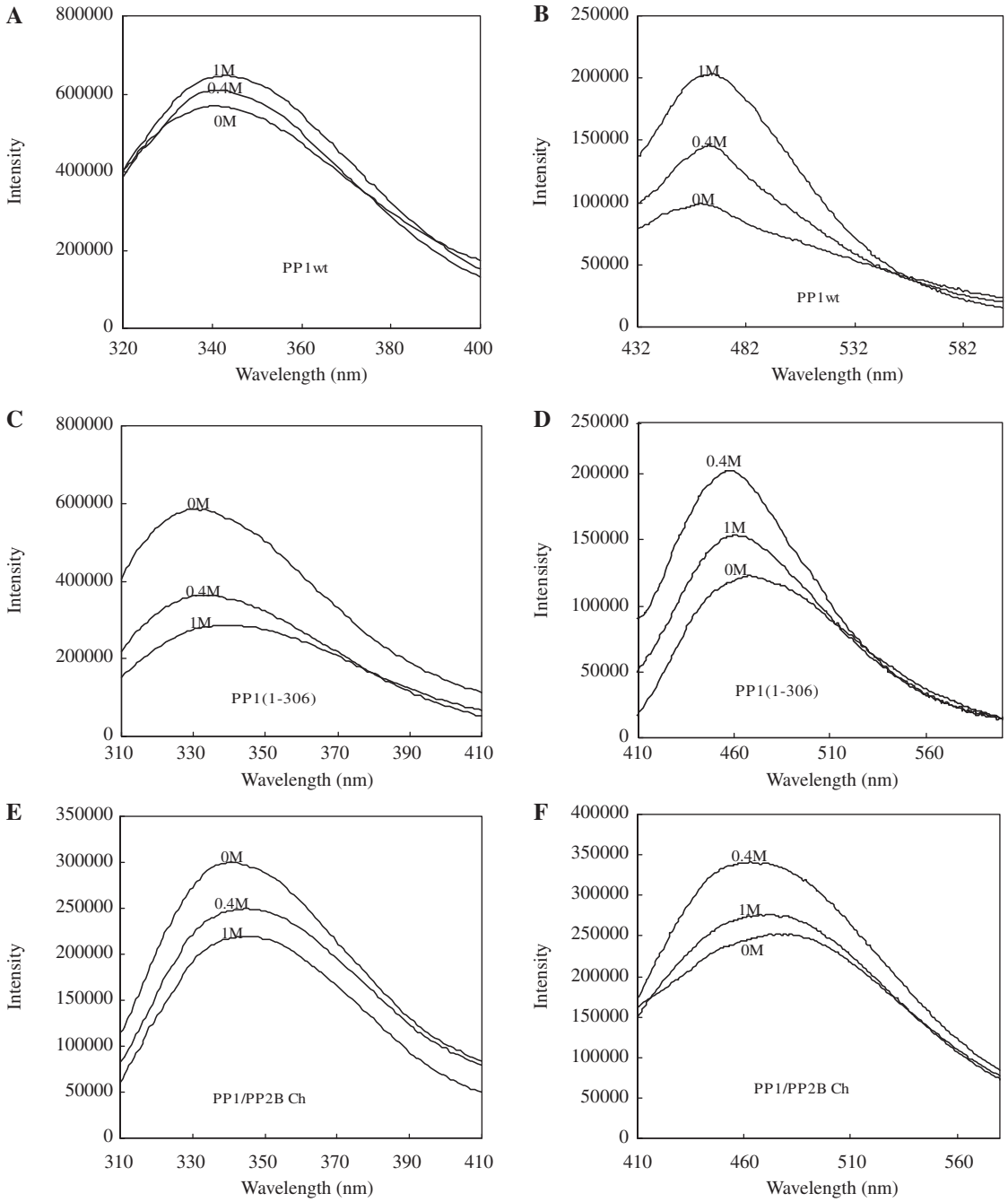

Fig. 4 Intrinsic and ANS fluorescence spectra of PP1wt and mutants before and after incubation with GdnHCl. (**A**) Intrinsic fluorescence spectra of PP1wt, (**B**) extrinsic fluorescence spectra of PP1wt, (**C**) intrinsic fluorescence spectra of PP1 (1–306), (**D**): extrinsic fluorescence spectra of PP1(1–306), (**E**) intrinsic fluorescence spectra of PP1/PP2B Ch and (**F**) extrinsic fluorescence spectra of PP1/PP2B Ch. For details see 'Materials and Methods' section.

Inhibition of phosphatase activity

Since PP1 is regulated by many inhibitors in vivo (*17*, *18*), we also examined the effects of several inhibitors on the deletion derivative and chimera of PP1 to gain some insight into the role of the C-terminus in interactions with the inhibitors. We measured inhibition by Microcystin-LR (MCLR), Tautomycin, Okadaic acid and Norcantharidin (Fig. 5 and Table2). The IC_{50} of MCLR for PP1 (1–306) was 2.5-fold higher than that for PP1wt while the IC_{50} of PP1/PP2B Ch was 2.5-fold lower than that of PP1wt (Fig. 5A). The IC_{50} values of TM and NCTD for PP1 (1–306) were similar to those for PP1wt whereas those of TM and NCTD for PP1/PP2B Ch were 4- and 2.5-fold higher, respectively than those for PP1wt (Fig. 5B and D). The effect of OA on PP1/PP2B Ch was similar to its effect on PP1wt (Fig. 5C) whereas its IC_{50} for PP1 (1–306) was lower than that for wt.

Effect of metal ions on phosphatase activity

There are two Mn^{2+} ions in the active centre of PP1 that play important roles in catalysis (*19*, *20*). PP1 is

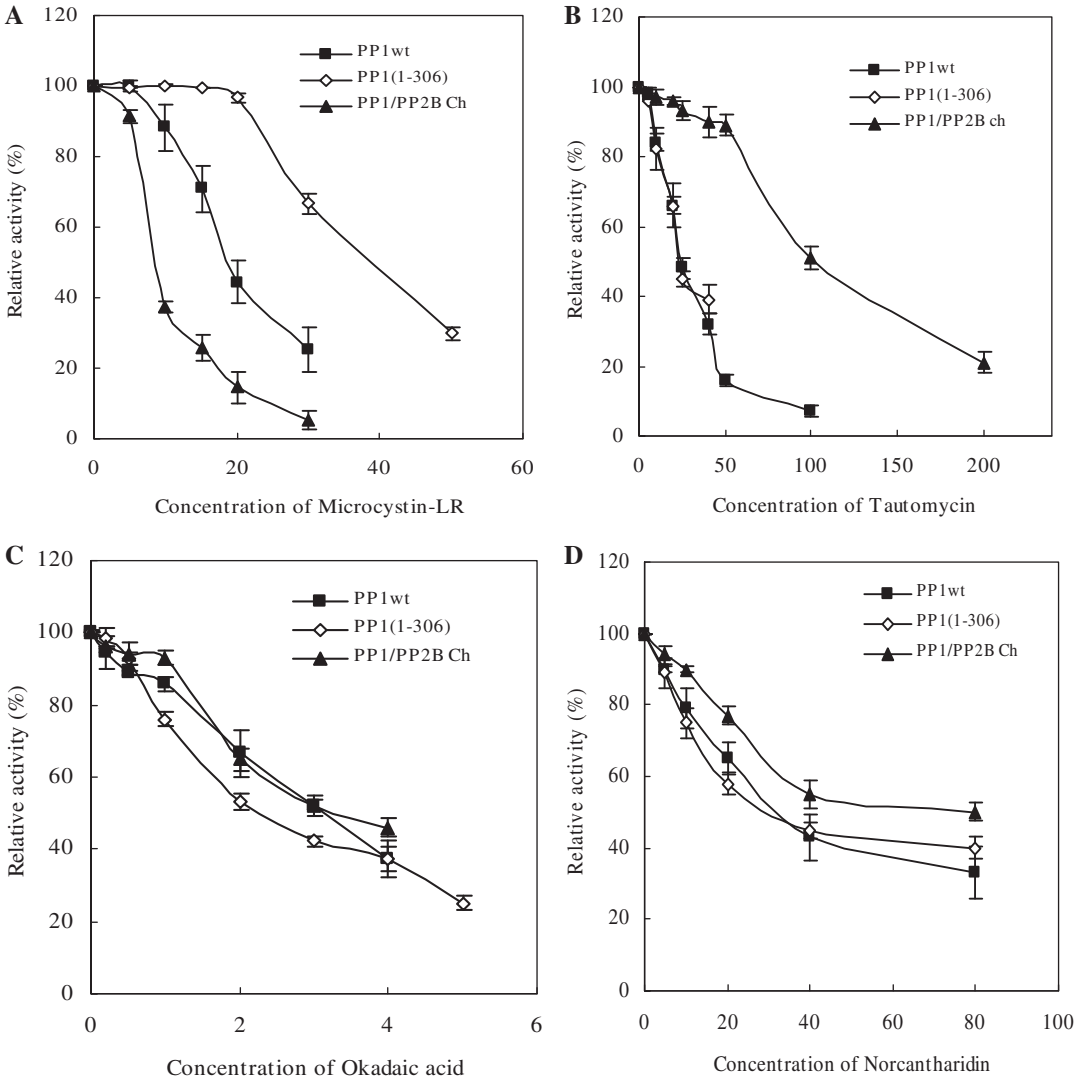

Fig. 5 Inhibition of PP1wt and mutants by protein phosphatase inhibitors. PP1wt (filled square), PP1(1–306) (open diamond) and PP1/PP2B Ch (filled triangle) are incubated with inhibitors at different concentrations and assayed using K-R-Tp-I-R-R as substrate. (**A**) inhibition by Microcystin-LR, (**B**) inhibition by Tautomycin, (**C**) inhibition by Okadaic acid and (**D**) inhibition by Norcantharidin. For details see 'Materials and Methods' section. Data are means ± SEM ($n \geq 3$).

Table II. Effects of toxins and inhibitor-2 on PP1wt and mutants.

	IC_{50}		
Inhibitors	**PP1wt**	**PP1(1–306)**	**PP1/PP2B Ch**
Microcystin-LR (nM)	18.0 ± 1.1	42.0 ± 1.4	8.0 ± 0.5
Tautomycin (nM)	28.2 ± 1.5	28.0 ± 1.0	106.0 ± 1.7
Okadaic acid (μM)	3.6 ± 0.1	2.2 ± 0.1	3.5 ± 0.1
Norcantharidin (μM)	34.5 ± 2.0	31.0 ± 0.9	80.0 ± 4.3

also dependent on external metal ions. We selected Mn^{2+}, Ni^{2+}, Mg^{2+}, Co^{2+} ions to explore effect on the wt and mutant enzymes (Fig. 6A–D). PP1wt was activated by Mn^{2+}, while other metal ions had no obvious effect. PP1(1–306) differed from PP1wt in that it was activated by Mn^{2+}, Ni^{2+}, Mg^{2+} to different degrees. These effects were similar to those observed with the CNAa, but the extent of activation of PP1 (1–306) was lower than that of CNAa (*20*). We also found that Mn^{2+}, $Mg^{2+}$and Co^{2+} clearly activated PP1/PP2B Ch, as they do the catalytic subunit of PP2B (CNA) (*21*). VO_4^{3-} did not activate PP1wt; it activated PP1-(1–306) to some extent and strongly activated PP1/PP2B Ch (Fig. 6E). We showed previously that VO_4^{3-} caused some activation of PP2B in this concentration range.

Discussion

PP1 and PP2B belong to the PPP family of Ser/Thr protein phosphatase whose members have highly homologous catalytic domains but not in N- and C-termini (*7*, *22*, *23*). The crystal structure of PP2B shows that its autoinhibitory domain interacts with its catalytic core, and the active site structure of PP2B is similar to that of PP1. In order to confirm whether PP1 has an inhibitory domain, and to confirm function of the

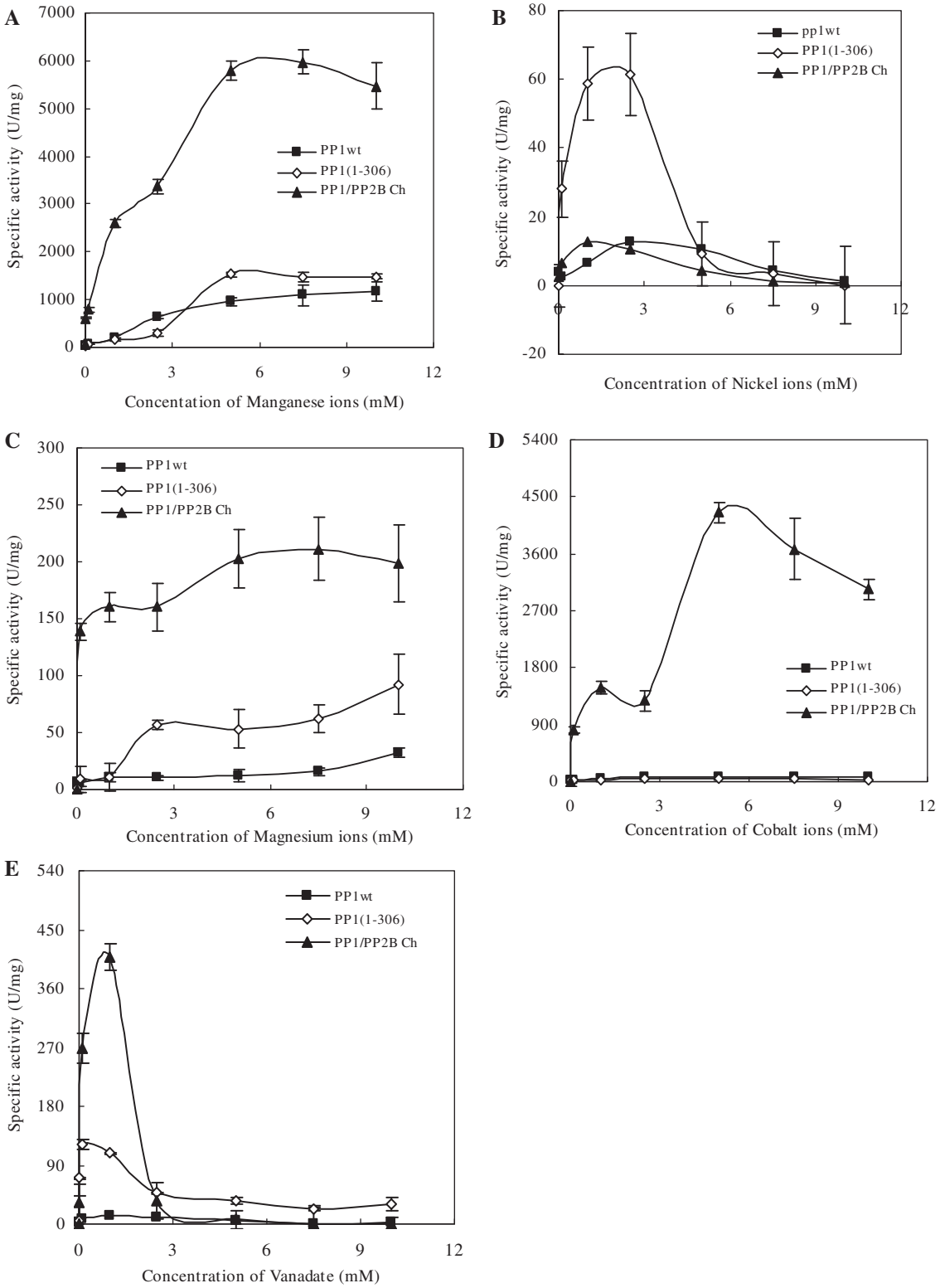

Fig. 6 Effect of metal ions on PP1wt and mutants. PP1wt (filled square), PP1-(1–306) (open diamond) and PP1/PP2B Ch (filled triangle) are incubated with different concentration of metal ions and assayed using K-R-Tp-I-R-R as substrate. (**A**) Mn^{2+}, (**B**) Ni^{2+}, (**C**) Mg^{2+}, (**D**) Co^{2+} and (**E**) VO_4^{3-}. For details see 'Materials and Methods' section. Each determination is performed in triplicate.

C-terminus (483–511 segments) of PP2B, we construct a deletion mutant PP1 (1–306) and a chimeric protein PP1/PP2B Ch. When the C-terminal 24 amino acids of PP1 are deleted, the enzymatic activity increases 3.5-fold using the substrate of K-R-Tp-I-R-R, and the enzymatic activity of PP1/PP2B Ch is even lower than that of PP1wt. In addition, the K_{cat} and K_{cat}/K_m of PP1(1–306) increase 16-fold, while that of PP1/PP2B Ch lower than that of PP1wt. These observations demonstrate that the C-terminus of PP2B

inhibits enzyme activity and are consistent with previous evidence that the whole C-terminus of PP2B (from CBD to C-terminus, 389–511) is an auto-inhibitory domain (*11*).

Observations on denaturation by GdnHCl show that deletion of the PP1 C-terminus results in a reduction in enzyme stability; importantly fusion with the PP2B C-terminus does not reverse this reduction. This indicates that although PP1 and PP2B have high sequence homology, their non-conserved termini may have different functions with respect to their conserved catalytic domains. In order to probe the structural basis of the reduced stability of the C-terminally deleted PP1 enzyme, we monitor changes of its conformation by examining fluorescence spectra. We find that the conformational changes of the mutants are different from those of PP1wt, and those of PP1 (1–306) are more marked than those of PP1/PP2B Ch. Moreover, the emission peak of PP1 (1–306) shows a clearcut blue shift in the absence of denaturant, demonstrating that there is a marked change in enzyme conformation. This change may 'loosen' the enzyme conformation and so facilitate the interaction with substrate molecules and increase the enzyme activity. Fusion with the C-terminus of PP2B appeared to make the enzyme more compact than PP1 (1–306) but not sufficiently so to significantly increase its stability in GdnHCl.

PP1 is regulated by many inhibitors *in vivo* (*16*, *24*, *25*). We select Microcystin-LR, Tautomycin, Norcantharidin and Okadaic acid to explore how deletion of the C-terminus and fusion with the PP2B C-terminus affect inhibitor interactions. We find that TM and NCTD have almost no influence on PP1-(1–306). The IC_{50} of MCLR for PP1-(1–306) increases 2.5-fold compared with that for PP1wt, whereas the IC_{50} values for TM and NCTD are essentially unchanged. This suggests that the C-terminus of PP1 does not participate in the interaction with TM and NCTD, and may interact weakly with MCLR. On the other hand, fusion with the C-terminus of PP2B reduced sensitivity to TM and NCTD, indicating that the C-terminus of PP2B has some blocking effect on the interaction with these inhibitors. As OA is a typical PP1 inhibitor, it is well known that the major site of interaction of these inhibitors is with the β12-β13 loop. Data showed that the IC_{50} of OA for PP1 (1–306) was lower than that for PP1wt; it means that the PP1 C-terminus itself may interfere with binding of OA. The C-terminus of PP2B has same effect with that of PP1 in interaction with OA.

In the study of enzyme activation by metal ions we find that PP1-(1–306) is activated in different extents by Ni^{2+}, Mg^{2+} and VO_4^{3-} different from PP1wt. Its behaviour is more similar to that of CNAa (*21*). It proved the fact that that PP1-(1–306) resembles closely the catalytic domain of protein phosphatases generally. It also implies that the interaction sites with metal ions are not located in the C-terminus of PP1. The metal activation data for the chimera PP1/PP2B Ch are similar to those for PP1-(1–306), but it was activated to a greater extent. PP1/PP2B Ch unlike PP1wt is clearly activated by VO_4^{3-} while PP1-(1–306) is only slightly activated. This means that the 483–511 segment of PP2B plays an important role in interactions with metal ions, although it is not an important functional domain.

In summary, this study demonstrates that the 483–511 segment of PP2B inhibits enzymatic activity and participates in activation by metal ions, and sometimes has an inhibitory effect on the interaction with various inhibitors. The C-terminus of PP1 also reduces enzymatic activity, but it stabilizes the enzyme conformation. In addition, it does not participate in interacting with metal ions and inhibitors TM and NCTD, but it may interact weakly with MCLR and may disturb the interaction with OA.

Acknowledgements

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Conflict of interest
None declared.

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