Biometals (2009) 22:469–477
DOI 10.1007/s10534-009-9204-9

Substrate-dependent metal preference of PPM1H, a cancer-associated protein phosphatase 2C: comparison with other family members

Takeyuki Sugiura · Yoshie Noguchi

Received: 29 October 2008 / Accepted: 1 January 2009 / Published online: 5 March 2009

Abstract Protein phosphatase 2C (PP2C) family is characterized by requirement of metal cation for phosphatase activity. We previously established that PPM1H is a cancer-associated member of the PP2C family. Here we further characterized the phosphatase activity of PPM1H, focusing on its dependence on metal cation. PPM1H possesses the potential to dephosphorylate *p*-nitrophenyl phosphate (pNPP), casein and phosphopeptides. Interestingly, PPM1H shows the metal preference that is varied depending on the substrate (substrate-dependent metal preference); PPM1H prefers Mn^{2+} when pNPP or phosphopeptides is used as a substrate. Meanwhile, a preference for Mg^{2+} is displayed by PPM1H with casein as a substrate. When both cations are added to the reaction, the degree of the effect is always closer to that with Mn^{2+} alone, irrespective of the substrate. This preponderance of Mn^{2+} is explained by its greater affinity for PPM1H than Mg^{2+}. From the literature the substrate-dependent metal preference appears to be shared by other PP2Cs. According to the crystal structure, a binuclear metal center of PP2C plays an important role for coordinating the substrate and nucleophilic waters in the active site. Therefore, the differences in the size, preferred geometry and coordination requirements between two metals, in relation to the substrate, may be responsible for this intriguing property.

Keywords Catalytic domain · Metal cation cofactor · PPM1H · Protein phosphatase 2C

T. Sugiura (✉) · Y. Noguchi
Discovery Research Laboratory, Tokyo R&D Center, Daiichi Pharmaceutical Co., Ltd., Daiichi-Sankyo Group, Tokyo, Japan
e-mail: sugiura.takeyuki.uu@daiichisankyo.co.jp

Present Address:
T. Sugiura
Exploratory Research Laboratories II, Kasai R&D Center, Daiichi-Sankyo Co., Ltd., 16-13, Kitakasai 1-Chome, Edogawa-ku, Tokyo 134-8630, Japan

Introduction

The reversible phosphorylation of proteins, controlled by protein kinases and phosphatases, is a critical mechanism by which eukaryotic organisms regulate cellular signal transduction pathways (Hunter 1995). Protein phosphatases are grouped into the protein tyrosine phosphatases and the protein serine/threonine phosphatases (PPs) on the basis of their substrate specificity. PPs are further divided into two large gene families, designated PPP (phosphoprotein phosphatase) and PPM (Mg^{2+}- or Mn^{2+}-dependent protein phosphatase) (Cohen 1992). Protein phosphatase 2C (PP2C) is a defining member of the PPM family, characterized by a monomeric form, dependence on divalent cations, and insensitivity to okadaic acid (Cohen 1992; Marley et al. 1996). The PP2C members are implicated in cancer-related biological processes such as stress responses,

apoptosis, and cell cycle (Tamura et al. 2006). For instance, the PPM1D gene is amplified and acts as an oncogene through negative feed-back regulation of p53 in several cancers (Bulavin et al. 2002).

In a series of our searches for new anticancer drug targets with the aid of the gene expression profile database (Sugiura et al. 2004, 2007, 2008a, b; Sugiura and Miyamoto 2008), we identified protein phosphatase 1H (PPM1H) containing the PP2C catalytic domain, upregulated in colon adenocarcinomas compared with normal colon tissues (Sugiura et al. 2008a). With *p*-nitrophenyl phosphate (pNPP) as a substrate, we demonstrated that recombinant PPM1H fused with maltose-binding protein (MBP-PPM1H) reveals the hallmarks of the PP2C family (Sugiura et al. 2008a). Furthermore, because PPM1H recognizes CSE1L, a proliferation and apoptosis-related protein, as a potential substrate and knockdown of its expression by specific short interference RNAs suppresses cell growth, PPM1H, too, is envisioned to be involved in cancer pathogenesis (Sugiura et al. 2008a).

As described above, metal cations are essential for PP2C activity, through bridging the amino acid residues of the PP2C active site to nucleophilic water molecules (Das et al. 1996). Despite the generally accepted view that the PP2C members require divalent metal cations such as Mg^{2+} or Mn^{2+} for their activity, physiological metal ions of PP2C are not known (Fjeld and Denu 1999). In addition, confusing results have been reported as PP2Cα activity was originally discovered as a Mg^{2+}-dependent casein phosphatase (McGowan and Cohen 1987) but in the other study only Mn^{2+} can support the pNPPase activity of PP2Cα (Marley et al. 1996). Although Mn^{2+} can be a surrogate of Mg^{2+} in the majority of enzymes, exchange between two metals may lead to some differences in biochemical interactions, thereby altering the properties of enzymes (Maguire and Cowan 2002). With these facts in mind, the deficiency in the detailed characterization of the PP2C enzymatic activity except for PP2Cα and the emerging significant roles of mammalian PP2Cs (Stern et al. 2007) further evoked our interest in the characters of the PP2C family.

Here we investigated in more detail the phosphatase activity of PPM1H as a model PP2C enzyme, with a focus on its metal requirement. As a result, we found that PPM1H shows metal preference for its activity, which varies according to the substrate. Further literature search unveiled that this substrate-dependent metal preference may prevail in the PP2C enzymes. The kinetic parameter analysis of PPM1H in this study adds to the existing biochemical data on the mammalian PP2C family essentially exclusive to PP2Cα. Moreover, the elucidation of substrate-dependent metal preference would provide foundation for a better understanding of the catalytic mechanism of PP2C enzymes.

Materials and methods

Preparation of recombinant PPM1H

MBP-PPM1H was expressed in *Escherichia coli* (*E. coli*) transformed with pMAL-c2X vector (New England Biolabs) harboring its sequence and purified near to homogeneity as described in (Sugiura et al. 2008a).

General phosphatase assay conditions

All the phosphatase assays were run in buffer A (40 mM Tris–HCl, pH 8.0, 20 mM KCl and 2 mM dithiothreitol) with the respective substrate (pNPP, casein or phoshpopeptide) and purified MBP-PPM1H as an enzyme source. Using mutant PPM1H, in which aspartic acid-437 was replaced by alanine, we corroborated that the phosphatase reaction of MBP-PPM1H toward pNPP hinges on the active site (Sugiura et al. 2008a). Each assay was repeated at least twice to confirm reproducibility. For calculation, the molecular weight of MBP-PPM1H and PP2Cα was assumed to be 100 and 42 kDa, respectively.

pNPPase activity

The activity of the enzyme with 5 mM pNPP (Sigma) as a substrate was assayed in 500 µl buffer A containing 1 µg enzyme with or without 10 mM Mn^{2+} and/or 30 mM Mg^{2+} at 30°C, following the previous experimental conditions for PP2Cα (Takai and Mieskes 1991; Marley et al. 1996). After 5 min, liberation of *p*-nitrophenol was determined spectrophotometrically at 405 nm.

Casein phosphatase activity

The activity of 1 µg enzyme was assayed toward 40 µg casein (Sigma) in 70 µl buffer A in the

presence or absence of 10 mM Mn^{2+} and/or Mg^{2+} at 37°C. Prior to the reaction free phosphate had been removed from casein dissolved in buffer A by filtration through YM-10 membrane (Millipore). About 60 min later, the reaction was stopped by the addition of 200 µl malachite green (Sigma) solution, which was prepared as described previously (Fathi et al. 2002). After 15 min at room temperature the amount of the released phosphate was evaluated by measuring the absorption at 650 nm. A standard curve was drawn using phosphate standard (K_3PO_4) for the determination of the phosphate amount.

Peptide phosphatase activity

The activity of the enzyme (1 µg) with phosphopeptide substrates (0.5 mM) was assayed in 70 µl buffer A with or without 10 mM Mn^{2+} and/or Mg^{2+} at 37°C. About 10 min later, release of phosphate was measured with the malachite green assay as above. Phosphopeptides were synthesized at toray research center (Kamakura, Kanagawa, Japan).

Determination of kinetic parameters

To measure kinetic constants for the metal ions, the assays were run in 70 µl buffer A under the conditions of 0.5 µg enzyme, 0.5 mM of the respective peptide and varying concentrations (0–50 mM) of each metal ion. When the kinetic parameters for phosphopeptides were measured, 0.5 µg enzyme, 10 mM Mn^{2+} and various concentrations (0–2,000 µM) of the respective peptide were included in the reaction. The phosphatase activity was assessed as described above for the peptide phosphatase activity. The kinetic parameters were determined by fitting the data to the Michaelis–Menten equation via KaleidaGraph (Synergy Software, Reading, PA).

Results

PPM1H requires Mn^{2+} when pNPP is used as a substrate

In the previous study we proved that the phosphatase activity of PPM1H toward pNPP is dependent on Mn^{2+} but did not examine the effect of an alternate cation, Mg^{2+} (Sugiura et al. 2008a). Hence, we first compared the effects of divalent metal cations, Mg^{2+} and Mn^{2+}, on the pNPPase activity of PPM1H. We used the preparation of PPM1H fused with MBP purified from the *E. coli* culture as PPM1H (Sugiura et al. 2008a). We selected MBP as a fusion tag after a few other tags were tested. We did not remove a MBP portion because it exhibited no phosphatase activity and was unexpected to disturb the phosphatase assay (Kashiwaba et al. 2003; Komaki et al. 2003). This is the case for many MBP fusions (Sachdev and Chirgwin 2000). As shown in Fig. 1, PPM1H hydrolyzed pNPP vigorously when Mn^{2+} was added at 10 mM to the reaction, whereas Mg^{2+} barely promoted the activity even at 30 mM. Thus, PPM1H needs Mn^{2+} for catalyzing pNPP. In the presence of both metals, the PPM1H fusion protein displayed the activity level similar to that exhibited in Mn^{2+} alone, indicating that the effect of Mn^{2+} dominates over that of Mg^{2+} as a cofactor under the experimental conditions.

Mg^{2+} is preferential for PPM1H activity with casein as a substrate

Since pNPP is not a native substrate, we next investigated the phosphatase activity of PPM1H toward a more natural substrate, phosphorylated amino acid residues of a protein. As a macromolecular substrate, we chose casein because its commercially available preparation is sufficiently phosphorylated without additional phosphorylation (Fathi et al. 2002). The amount of the released phosphate was determined by the sensitive malachite green assay (Fathi et al. 2002). Intriguingly, MBP-PPM1H could liberate phosphates

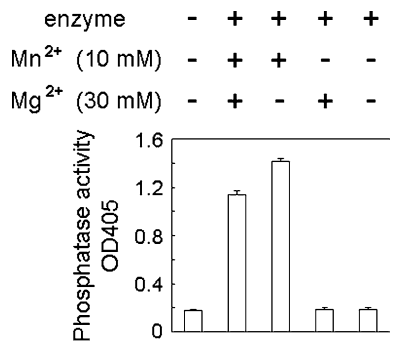

Fig. 1 Metal cation effects on PPM1H phosphatase activity toward pNPP. Divalent cations (30 mM Mg^{2+} and/or 10 mM Mn^{2+}) were added to the reactions as indicated. Each assay was done in duplicate and the average values are indicated with standard deviations

of casein remarkably with Mg^{2+} but only marginally with Mn^{2+} at their concentrations of 10 mM (Fig. 2), which was the opposite tendency to the case where pNPP served as a substrate. When both Mg^{2+} and Mn^{2+} existed in the reaction, the activity was reduced to the level similar to that obtained solely with Mn^{2+} (Fig. 2). Again, the influence of Mn^{2+} on the PPM1H's phosphatase activity overrides that of Mg^{2+}.

Mn^{2+} is optimal for PPM1H activity toward phosphorylated peptides

It is noteworthy that among PPs the activity of PP2A and PP2C toward short phosphopeptides is high while that of PP1 and PP2B is minimal (Pinna and Donella-Deana 1994). We were interested to determine whether PPM1H could dephosphorylate peptide substrates. We prepared three peptides that were commonly utilized for the analysis of protein phosphatases (Pinna and Donella-Deana 1994). Assays of PPM1H using these phosphopeptides showed that among them, RRA-pT-VA was the most favorable substrate for the phosphatase (Fig. 3). RRA-pT-PA was hardly susceptible to the PPM1H activity, as in the case of other PP2C members (Pinna and Donella-Deana 1994). Both Mg^{2+} and Mn^{2+} enhanced the PPM1H-elicited phosphate hydrolysis of two peptide substrates substantially at 10 mM. Yet Mn^{2+} was obviously a more potent activator compared with Mg^{2+}. Consequently, PPM1H prefers Mn^{2+} when phosphopeptides are used

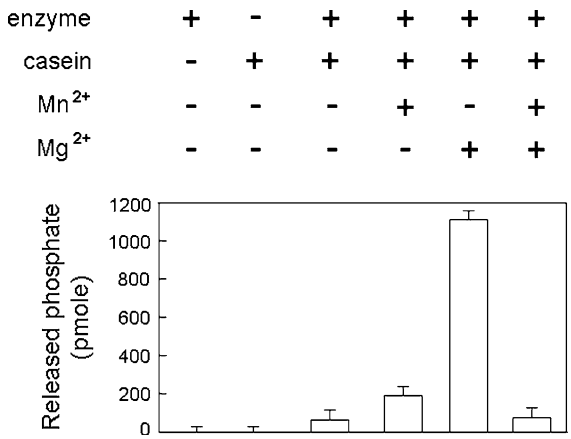

Fig. 2 Phosphatase activity of PPM1H with casein as a substrate. Phosphatase assays were carried out in the presence or absence of 10 mM metal ion(s) as indicated. Each assay was done in triplicate and the average amounts of released phosphate are indicated with standard deviations

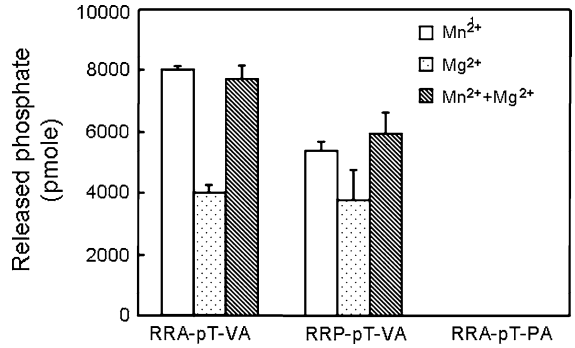

Fig. 3 Peptide substrate specificity of PPM1H. Phosphatase assays were conducted in buffer A containing MBP-PPM1H, the indicated phosphopeptide and 10 mM metal ion(s). Experiments were done in duplicate and the average amounts of released phosphate are depicted with standard deviations. There was essentially no activity of PPM1H toward any phosphopeptide without metal ion

as substrates. When a mixture of the metal cations was added, the degree of the activation was closer to that given by Mn^{2+} as the only cofactor. Combined with the above results, when both cations are present, the effect of Mn^{2+} on PPM1H is predominant over that of Mg^{2+}, regardless of the substrate.

Determination of kinetic parameters

Since either metal was able to induce the considerable level of the PPM1H activity for two phosphopeptides, we calculated kinetic parameters for the metals by using these peptides as substrates, assuming that metal ions are pseudo-substrates for PP2C (Fjeld and Denu 1999). The apparent K_{metal} values were smaller for Mn^{2+} than for Mg^{2+} with either of the peptides (Table 1), ensuring that Mn^{2+} binds PPM1H with a greater affinity than Mg^{2+}. This result is in line with the above notion that Mn^{2+} is a dominant cofactor

Table 1 Kinetic parameters of PPM1H for the metal cofactors with the indicated phosphopeptides as substrates

Metal	Peptide	V_{max} (μmol min^{-1} mg^{-1})	K_{metal} (mM)	V_{max}/K_{metal} (×1,000)
Mn^{2+}	RRA-pT-VA	0.40	7.4	53.9
	RRP-pT-VA	0.29	7.7	38.9
Mg^{2+}	RRA-pT-VA	0.46	29.9	13.3
	RRP-pT-VA	0.42	>50	<8.4

Assays were run in duplicate and the average values were used for the determination of the kinetic parameters

over Mg^{2+}. We further compared the phosphatase activity between PPM1H and a representative PP2C member, PP2Cα by determining the kinetic constants for the phosphopeptides. As Table 2 indicates, PPM1H showed elevated K_m and depressed K_{cat} for RRP-pT-VA relative to those for RRA-pT-VA, confirming that the latter peptide is a favorable substrate for PPM1H. The K_m value of PPM1H for a typical phosphopeptide, RRA-pT-VA, was comparable with that of PP2Cα while the K_{cat} of PPM1H was greatly lowered (Table 2). In conclusion, the variations in the sequence of catalytic motifs between PP2Cα and PPM1H (Fig. 4) modulate the catalytic efficiency, but not the affinity, for the phosphorylated peptide substrate. Nevertheless, these PP2C enzymes exhibit a similar pattern of the substrate-dependent metal preference (see below).

Table 2 Kinetic parameters for PP2Cα and PPM1H with phosphopeptide substrates

Peptide	V_{max} (μmol min^{-1} mg^{-1})	K_{cat} (s^{-1})	K_m (μM)	$K_{cat}/K_m \times 10^3$ (s^{-1} M^{-1})
(PP2Cα)				
RRA-pT-VA	23.5	16.5	152	109
(PPM1H)				
RRA-pT-VA	1.01	1.7	159.7	10.6
RRP-pT-VA	0.54	0.9	217.7	4.1

Kinetic constants for PPM1H were determined by the phosphatase assays done in duplicate. The parameters for PP2Cα are derived from (Marley et al. 1996). Note that the assays were conducted at 30°C for PP2Cα and at 37°C for PPM1H. Therefore, the differences in V_{max} and, accordingly, K_{cat}/K_m between the phosphatases would be underestimated

Discussion

PP2C enzymes require metal ions for their phosphatase activity. The crystal structure of PP2Cα indicates that in the catalytic site there are two metal ions jointly hexacoordinated by oxygen atoms from protein residues and attacking water molecules. From this structure it is proposed that catalysis proceeds by direct attack of an activated water molecule at the phosphorus center of substrate (Das et al. 1996). Based on the well-conserved amino acid residues participating in coordination of metals and waters between PP2C enzymes (Bork et al. 1996; Das et al. 1996), the catalytic mechanism would likely be applicable to all these members.

We demonstrated here that besides pNPP (Sugiura et al. 2008a), PPM1H is capable of dephosphorylating casein and phosphopeptides. As a member of the PP2C family, PPM1H relies on metal cations for its catalytic action. But, importantly, the activity displays the metal preference that is varied depending on the substrate (substrate-dependent metal preference). When PPM1H catalyzes an artificial substrate, pNPP, only Mn^{2+} functions as an effective activator (Fig. 1).

	motif I		motif II		motif III		motif IV	
hPPM1H	88	KSTHNED	146	YWSLFDGHAG	268	GCTALIVICLLGKLYVANAGDSRAII	381	RVMATIGVTRGLGDH
hPP2Cα	33	RVEMED	55	FFAVYDGHAG	126	GSTAVGVLISPQHTYFINCGDSRGLL	186	RVNGSLAVSRALGDF
bPP2Cβ	33	RVEMED	55	FFAVYDGHAG	131	GSTAVGVMISPKHIYFINCGDSRAVL	191	RVNGSLAVSRALGDY
hPP2Cγ	36	RVSMED	55	MFSVYDGHGG	328	GTTAVVALIRGKQLIVANAGDSRCVV	399	RVNGGLNLSRAIGDH
rPP2Cδ	118	REEMQD	147	YFAVFDGHGG	216	GSTATCVLAVDNILYIANLGDSRAIL	282	RVLGVLEVSRSIGDG
mPP2Cη	25	KSEFNED	66	YWALFDGHGG	161	GCTALVAVFLQGKLYVANAGDSRAIL	274	RLLGTLAVSRGLGDH
mPP2Cζ	114	KSRHNED	154	YWGLFDGHAG	260	GCCALVVLYLLGKMYVANAGDSRAII	373	RVMATIGVTRGLGDH

	motif V		motif VI		catalytic motifs identity	accession No.
hPPM1H	414	PEVRIYDLSKYDHGSDDVLILATDGLWD	498	DDISVYVIPL		XM_051093
hPP2Cα	219	PEVHDIERSEEDDQFIILACDGIWD	282	DNMSVILICF	44.9%	AF070670
bPP2Cβ	224	PEVYEILRAEEDEFIILACDGIWD	286	DNMSIVLVCF	42.7%	NM_174430
hPP2Cγ	413	PDIKVLTLTDDHEFMVIACDGIWN	496	DNMTCIIICF	37.1%	NM_002707
rPP2Cδ	307	PDIRRCQLTPNDRFILLACDGLFK	381	DNVTVMVVRI	47.0%	AF095927
mPP2Cη	307	PQVTVLDVHQLAVQEEDVVVMATDGLWD	387	DDVSVFVIPL	64.6%	AY332616
mPP2Cζ	406	PEVRVYDLTQYEHCPDDVLVLGTDGLWD	490	DDISVFVIPL	82.5%	AY184802

Fig. 4 Sequence alignment of PPM1H with other human PP2C family members. The sequence of PPM1H was compared with that of other PP2Cs whose metal preferences are documented in the literature within the six conserved motifs (Komaki et al. 2003). The *number* on the *left* denotes the amino acid position for each protein. Prefixes, h, b, r, and m, decipher human, bovine, rat and mouse, respectively. The sequence identity within the catalytic motifs between PPM1H and the other members was calculated with Genetyx software (Software Development Co., Ltd., Tokyo, Japan)

The incapability of hydrolyzing pNPP with Mg^{2+} as a coactivator has also been reported for an archetypal PP2C, PP2Cα (Marley et al. 1996). For substrate containing innate structures (phosphorylated threonine and serine), the metal preference is also evident as Mg^{2+} gives higher activity toward a casein substrate (Fig. 2) while Mn^{2+} is optimal for dephosphorylating phosphopeptides by PPM1H (Fig. 3).

Despite similar chemical characteristics between two metals, Mn^{2+} is significantly larger than Mg^{2+} (Baldwin et al. 1999). Additionally, Mn^{2+} is distinguished from Mg^{2+} in that Mn^{2+} has relaxed coordination requirements, allowing phosphoryl transfer reactions to occur with altered catalytic residues, non-ideal substrates, and reduced specificity (Yang et al. 2006). For example, restriction enzyme *EcoR*V gains the ability to cut noncognate sites with Mn^{2+} in place of Mg^{2+} as a cofactor (Vermote and Halford 1992). Within the confines of an active site, only the metal ion with the appropriate size and the coordination geometry would be able to properly position the substrate and the nucleophiles. In this sense, it is not unexpected that PPM1H, classified as a serine/threonine phosphatase, is unable to accommodate bulky pNPP akin to that of a tyrosyl residue of phosphotyrosine in a preferable geometry, thereby, failing to recognize it as a substrate with Mg^{2+}. In the meantime, the tolerant feature of Mn^{2+} could realize adequate positioning of pNPP in the active site of PPM1H, leading to substrate hydrolysis. We reasoned that Mg^{2+} permits phosphorylated serine or threonine residues of a rather rigid protein substrate, casein to be accommodated in the catalytic site, whereas a larger size of Mn^{2+} perturbs the delicate geometry in the active site compatible with catalytic activity, resulting in the reduced phosphatase activity of PPM1H. On the other hand, non-native but flexible peptide substrates should not necessitate structural adjustments on the part of the enzyme even with a larger metal, Mn^{2+}. In this setting the artificial nature of peptides may prefer Mn^{2+} harboring relaxed requirements for coordination. In any case, given that the substrate-dependent metal preference is offered by the differences between Mg^{2+} and Mn^{2+}, we assume that the balance between the atomic size and the preferred geometry of the metal, in relation to the substrates, would contribute to the substrate-dependent metal preference.

In the literature other members of the mammalian PP2C family provide varied patterns of the metal ion requirement for their enzymatic activity, as shown in Table 3. The substrate-dependent metal preference is observed, at least, for PP2Cα and PP2Cβ as well. From these observations, it is conceivable that this property is shared by other PP2Cs. Then, care must be taken when metal requirement of PP2C enzymes is discussed as it might be variable depending on the individual substrate. PP2Cα prefers Mn^{2+} when the substrate is pNPP, whereas Mg^{2+} is favorable with casein as a

Table 3 Comparison of the metal preference among PP2C family members

PP2C	Substrate	Metal preference	Reference
hPPM1H	pNPP	Mn^{2+}	This study
	Phosphopeptides	Mn^{2+}	This study
	Casein	Mg^{2+}	This study
hPP2Cα	pNPP	Mn^{2+}	Marley et al. (1996)
	Casein	Mg^{2+}	Kashiwaba et al. (2003)
bPP2Cβ	Phosphopeptide	Mg^{2+}	Krieglstein et al. (2003)
	Casein	Mn^{2+}	Krieglstein et al. (2003)
hPP2Cγ	MyBP[a]	Mn^{2+}	Travis and Welsh (1997)
rPP2Cδ	MyBP	Mn^{2+}	Tong et al. (1998)
mPP2Cη	Casein	Mn^{2+}	Komaki et al. (2003)
mPP2Cζ	Casein	$Mg^{2+} \approx Mn^{2+}$	Kashiwaba et al. (2003)

PP2C enzymes shown in Fig. 4 are compiled for comparison in terms of the substrate-dependent metal preference. Prefixes are used as in Fig. 4. It should be noticed that the assay conditions for the respective substrate were not necessarily the same in the different studies dealing with various PP2Cs. Therefore, we cannot completely exclude the possibility of a misleading conclusion drawn from this table

[a] Myelin basic protein

substrate (Table 3). Accordingly, the substrate-dependent metal preference is retained between PPM1H and PP2Cα though these PP2Cs reveal a relatively low sequence identity in the catalytic motifs (Fig. 4). In fact, PPM1H and PP2Cα are categorized into different subgroups of the PP2C family (Stern et al. 2007). The differences in the catalytic motif sequences between two PP2Cs affect the catalytic efficiency (Table 1) but not either the affinity for the peptide substrate (Table 2) or the substrate-dependent metal preference (Table 3), inferring that the substrate-dependent metal preference is more closely associated with an initial binding stage than a catalytic stage, which is governed by a chemical step and product release through both the orientations and juxtapositions of the involved atoms (Adams and Taylor 1993; Cannon et al. 1996). Thus, the substrate-dependent metal preference could be dictated by a manner in which metal cations and a substrate are cooperatively lodged into the enzyme's active site. Furthermore, PP2C binds metal cations before the substrate (Fjeld and Denu 1999). In view of the ordered sequence of their binding to the enzyme, it would be a metal ion rather than a substrate that is an actual determiner of the preference.

Conversely, although the sequence of PPM1H within the catalytic motifs is highly similar to that of PP2Cζ and η (Fig. 4), unlike PPM1H, the activity of the former PP2C toward casein is indistinguishable with either metal ion (Kashiwaba et al. 2003), whereas the latter achieves a greater phosphatase activity toward casein with Mn^{2+} (Komaki et al. 2003). Although it is reasonable to hypothesize that the size, preferred geometry and coordination requirements would determine the metal preference, the priority of these factors would vary depending on the relationship between the individual PP2C members and the different substrates. In this case, the sequence similarity in the active site motifs correlates with the metal preference within these PP2Cs belonging to the same subgroup (Stern et al. 2007), suggesting that the priority of the factors for the metal preference is changed in accordance with the difference in the active site conformation. Further, despite the considerably high sequence identity within the active site motifs of PPM1H with PP2Cζ and η (Fig. 4), the entire sequence identity is just moderate (48.2% with PP2Cζ and 40.6% with PP2Cη). Hence it is also possible that the sequence outside the catalytic site motifs indirectly affects the configuration of the active site of these enzymes, eventually leading to the variation of the metal preference greater than expected.

For all the substrates, the effect of Mn^{2+} on PPM1H dominates over that of Mg^{2+} when both metals coexist (Figs. 1, 2, 3). This preponderance can simply be recognized as a reflection of the greater affinity of Mn^{2+} for PPM1H relative to Mg^{2+}, as evidenced from the smaller K_{metal} value for Mn^{2+} (Table 1). The greater affinity of Mn^{2+} in comparison with Mg^{2+} is common with binuclear metallohydrolases, PP2Cα, *EcoR*V and RNase H (Vermote and Halford 1992; Wilcox 1996; Goedken and Marqusee 2001), which might be ascribed to the stronger electronegativity of Mn^{2+} (Baldwin et al. 1999).

In the present study we must use unphysiologically high concentrations of metal ions for the phosphatase activity of PPM1H, as has also been the case for other PP2C members (Marley et al. 1996; Travis and Welsh 1997; Tong et al. 1998; Kashiwaba et al. 2003; Komaki et al. 2003; Krieglstein et al. 2003). Although mammalian PP2Cs are believed to have two metal binding sites as described above, there is an additional metal binding site with low affinity in bacterial PP2C, of which role in catalysis is controversial (Wehenkel et al. 2007). As the amino acid residues forming the third metal binding site are conserved in mammalian PP2C as well (Schlicker et al. 2008), the third metal may bind to the catalytic site of PPM1H at its high concentrations, influencing the property of the enzyme in some way. In vivo, unsaturated fatty acids may augment the activity of PP2Cs synergistically with a lowered concentration of metal ions (Krieglstein et al. 2003). We cannot exclude the possibility that the substrate-dependent metal preference is a consequence of the high concentrations (relative to in vivo levels) of metal cations used in our experiments. Nonetheless, the substrate-dependent metal preference is of importance because the study of this phenomenon could provide information on the electronic, substrate structural and catalytic requirements for the enzymatic process of this phosphatase family (Weber et al. 2003). Regarding this respect, structural determination of mammalian PP2C enzymes other than PP2Cα (Das et al. 1996) and comparison of their structures in complex with metal ion and different substrates would test the hypotheses described herein and are fundamental to understanding the molecular basis of the substrate-dependent metal preference.

Acknowledgments We thank Y. Nagano for technical assistance and Dr. J. Hasegawa for critical reading of the manuscript.

References

Adams JA, Taylor SS (1993) Divalent metal ions influence catalysis and active-site accessibility in the cAMP-dependent protein kinase. Protein Sci 2:2177–2186

Baldwin GS, Sessions RB, Erskine SG, Halford SE (1999) DNA cleavage by the *EcoR*V restriction endonuclease: roles of divalent metal ions in specificity and catalysis. J Mol Biol 288:87–103. doi:10.1006/jmbi.1999.2672

Bork P, Brown NP, Hegyi H, Schultz J (1996) The protein phosphatase 2C (PP2C) superfamily: detection of bacterial homologues. Protein Sci 5:1421–1425

Bulavin DV, Demidov ON, Saito S, Kauraniemi P, Phillips C, Amundson SA, Ambrosino C, Sauter G, Nebreda AR (2002) Amplification of PPM1D in human tumors abrogates p53 tumor-suppressor activity. Nat Genet 31:210–215. doi:10.1038/ng894

Cannon WR, Singleton SF, Benkovic SJ (1996) A perspective on biological catalysis. Nat Struct Biol 3:821–833. doi:10.1038/nsb1096-821

Cohen P (1992) Signal integration at the level of protein kinases, protein phosphatases and their substrates. Trends Biochem Sci 17:408–413. doi:10.1016/0968-0004(92)90010-7

Das AK, Helps NR, Cohen PT, Barford D (1996) Crystal structure of the protein serine/threonine phosphatase 2C at 2.0 a resolution. EMBO J 15:6798–6809

Fathi AR, Krautheim A, Lucke S, Becker K, Juergen-Steinfelder H (2002) Nonradioactive technique to measure protein phosphatase 2A-like activity and its inhibition by drugs in cell extracts. Anal Biochem 310:208–214. doi:10.1016/S0003-2697(02)00377-9

Fjeld CC, Denu JM (1999) Kinetic analysis of human serine/threonine protein phosphatase 2Cα. J Biol Chem 274:20336–20343. doi:10.1074/jbc.274.29.20336

Goedken ER, Marqusee S (2001) Co-crystal of *Escherichia coli* RNase HI with Mn^{2+} ions reveals two divalent metals bound in the active site. J Biol Chem 276:7266–7271. doi:10.1074/jbc.M009626200

Hunter T (1995) Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. Cell 80:225–236. doi:10.1016/0092-8674(95)90405-0

Kashiwaba M, Katsura K, Ohnishi M, Sasaki M, Tanaka H, Nishimune Y, Kobayashi T, Tamura S (2003) A novel protein phosphatase 2C family member (PP2Cζ) is able to associate with ubiquitin conjugating enzyme. FEBS Lett 538:197–202. doi:10.1016/S0014-5793(03)00153-4

Komaki K, Katsura K, Ohnishi M, Li MG, Sasaki M, Watanabe M, Kobayashi T, Tamura S (2003) Molecular cloning of PP2Cη, a novel member of the protein phosphatase 2C family. Biochim Biophys Acta 1630:130–137

Krieglstein J, Selke D, Maassen A, Klumpp S (2003) Activity of PP2Cβ is increased by divalent cations and lipophilic compounds depending on the substrate. Methods Enzymol 366:282–289. doi:10.1016/S0076-6879(03)66021-0

Maguire ME, Cowan JA (2002) Magnesium chemistry and biochemistry. Biometals 15:203–210. doi:10.1023/A:1016058229972

Marley AE, Sullivan JE, Carling D, Abbott WM, Smith GJ, Taylor IW, Carey F, Beri RK (1996) Biochemical characterization and deletion analysis of recombinant human protein phosphatase 2Cα. Biochem J 320:801–806

McGowan CH, Cohen P (1987) Identification of two isoenzymes of protein phosphatase 2C in both rabbit skeletal muscle and liver. Eur J Biochem 166:713–721. doi:10.1111/j.1432-1033.1987.tb13570.x

Pinna LA, Donella-Deana A (1994) Phosphorylated synthetic peptides as tools for studying protein phosphatases. Biochim Biophys Acta 1222:415–431. doi:10.1016/0167-4889(94)90050-7

Sachdev D, Chirgwin JM (2000) Fusions to maltose-binding protein: control of folding and solubility in protein purification. Methods Enzymol 326:312–321. doi:10.1016/S0076-6879(00)26062-X

Schlicker C, Fokina O, Kloft N, Grüne T, Becker S, Sheldrick GM, Forchhammer K (2008) Structural analysis of the PP2C phosphatase tPphA from *Thermosynechococcus elongatus*: a flexible flap subdomain controls access to the catalytic site. J Mol Biol 376:570–581. doi:10.1016/j.jmb.2007.11.097

Stern A, Privman E, Rasis M, Lavi S, Pupko T (2007) Evolution of the metazoan protein phosphatase 2C superfamily. J Mol Evol 64:61–70. doi:10.1007/s00239-006-0033-y

Sugiura T, Miyamoto K (2008) Characterization of TRIM31, upregulated in gastric cancer, as a novel RBCC protein. J Cell Biochem 105:1081–1091. doi:10.1002/jcb.21908

Sugiura T, Nagano Y, Inoue T, Hirotani K (2004) A novel mitochondrial C_1-tetrahydrofolate synthetase is upregulated in human colon adenocarcinoma. Biochem Biophys Res Commun 315:204–211. doi:10.1016/j.bbrc.2004.01.035

Sugiura T, Nagano Y, Noguchi Y (2007) DDX39, upregulated in lung squamous cell cancer, displays RNA helicase activities and promotes cancer cell growth. Cancer Biol Ther 6:957–964. doi:10.1158/1535-7163.MCT-06-0634

Sugiura T, Noguchi Y, Sakurai K, Hattori C (2008a) Protein phosphatase 1H is overexpressed in colon adenocarcinoma and associated with CSE1L. Cancer Biol Ther 7:285–292

Sugiura T, Yamaguchi A, Miyamoto K (2008b) A cancer-associated RING finger protein, RNF43, is a ubiquitin ligase that interacts with a nuclear protein, HAP95. Exp Cell Res 314:1519–1528. doi:10.1016/j.yexcr.2008.01.013

Takai A, Mieskes G (1991) Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases. Biochem J 275:233–239

Tamura S, Toriumi S, Saito J, Awano K, Kudo TA, Kobayashi T (2006) PP2C family members play key roles in regulation of cell survival and apoptosis. Cancer Sci 97:563–567. doi:10.1111/j.1349-7006.2006.00219.x

Tong Y, Quirion R, Shen S-H (1998) Cloning and characterization of a novel mammalian PP2C isozyme. J Biol Chem 273:35282–35290. doi:10.1074/jbc.273.52.35282

Travis SM, Welsh MJ (1997) PP2Cγ: a human protein phosphatase with a unique acidic domain. FEBS Lett 412:415–419. doi:10.1016/S0014-5793(97)00837-5

Vermote CL, Halford SE (1992) *EcoR*V restriction endonuclease: communication between catalytic metal ions and DNA recognition. Biochemistry 31:6082–6089. doi:10.1021/bi00141a018

Weber TP, Widger WR, Kohn H (2003) Metal dependency for transcription factor rho activation. Biochemistry 42:1652–1659. doi:10.1021/bi020601y

Wehenkel A, Bellinzoni M, Schaeffer F, Villarino A, Alzari PM (2007) Structural and binding studies of the three-metal center in two mycobacterial PPM Ser/Thr protein phosphatases. J Mol Biol 374:890–898. doi:10.1016/j.jmb.2007.09.076

Wilcox DE (1996) Binuclear metallohydrolases. Chem Rev 96:2435–2458. doi:10.1021/cr950043b

Yang W, Lee JY, Nowotny M (2006) Making and breaking nucleic acids: two-Mg^{2+}-ion catalysis and substrate specificity. Mol Cell 22:5–13. doi:10.1016/j.molcel.2006.03.013