

Substrate Preference of p53-inducible Protein Phosphatase PPM1D

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Introduction

The regulation of cellular processes by the modulation of protein phosphorylation/dephosphorylation is fundamental to a large number of signal transduction pathways. Serine/threonine phosphatases are classified into two different families: the phosphoprotein phosphatases (PPP) and the phosphoprotein phosphatase M family (PPM) [1]. PPM1 type protein phosphatases are characterized by dependence on Mn^{2+} or Mg^{2+} for their activities and by insensitivity to okadaic acid which inhibit most PPP-type protein phosphatases [2, 3]. Different sensitivities against small inhibitors have indicated that the catalytic sites in the PPM1 phosphatases are shallower than those of their PPP counterparts, and therefore that small inhibitors cannot easily bind to the substrate-binding pockets of the PPM1 type phosphatases.

PPM1 type protein phosphatase PPM1D is induced in response to DNA damage by ultraviolet (UV) and infrared (IR) stimulation [4]. Recently, many other tumor suppressor proteins, such as Chk1, ATM, p38 and p53 itself have been identified as targets of PPM1D and these proteins were inactivated by the resulting dephosphorylation [5, 6]. The gene amplification of PPM1D was observed in several human cancers such as breast cancers. In addition, PPM1D-deficient mice and cells derived from them show a tumor-resistant phenotype, further suggesting that PPM1D plays a role as an oncogenic protein. These findings suggest that PPM1D is a viable anti-cancer target for drug development, but little is known about the specific inhibitor of this phosphatase.

Results and Discussion

It has been reported that substrate preference of PPM1D is different from another of the PPM1 type phosphatases, PPM1A, by *in vitro* phosphatase analysis, although their fundamental structures would be expected to be conserved in these two enzyme [7, 8]. To identify the residues within the catalytic center of PPM1D and thus further understand its substrate recognition mechanism, the primary structure of this phosphatase in human was aligned with other human PPM1-type phosphatases using Clustal W. The results of this analysis indicated that most of the metal chelating residues of PPM1D are well aligned with those of the other PPM1 family members. In support of the results of this amino acid alignment, a D314 mutant of PPM1D has been reported to act as a phosphatase-deficient protein [6]. On the other hand, a putative phosphate binding residue and two other metal chelating residues in the N-terminus of PPM1D did not align in our analysis. A refinement of this alignment among the PPM1 type protein phosphatases was then carried out to take account of long inserts that were clearly evident by eye [9]. This refined alignment revealed that

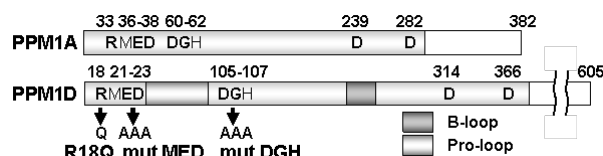


Fig. 1. Schematic diagrams of PPM1A and PPM1D. The wild-type and a series of mutants of PPM1D derivatives covered catalytic domain of PPM1D (1-420).

PPM1 family shares seven conserved regions. Region I contains putative phosphate binding site and two other metal chelating residues of PPM1D, *i.e.* R18 or K19 in PPM1D may work as phosphate binding residue. The residues of E22 and D23 in PPM1D were corresponding to E37, D38 residues of human PPM1A, respectively (Fig. 1).

Furthermore, the sequence alignment of PPM1D with other PPM1-type phosphatases also revealed that PPM1D contains two unique loops, a Pro-residue rich loop and a basic-residue rich loop designated the B-loop (Fig. 1).

To further evaluate whether the predictions resulting from our sequence alignment analysis of PPM1D were valid, and thus that the putative phosphate binding and metal-chelating residues indeed functioned in the catalytic site of this enzyme, we performed phosphatase analysis using PPM1D mutants.

We first performed a phosphatase assay using a recombinant PPM1D catalytic domain, designated PPM1Dc, comprising 420 residues. PPM1Dc was analyzed in a phosphatase activity assay using a human p53-derived peptide phosphorylated at position 15, p53(10-23)15P (WT), as a wild type substrate analog. In our current phosphatase assays using PPM1D mutants, the substitution of the well-conserved metal-chelating residues ¹⁰⁵DGH¹⁰⁷ with an Alanyl-alanyl-alanine moiety, mut-DGH (Fig. 1), completely abolished phosphatase activity. Similar to mut-DGH, the mut-MED protein harbouring a mutation in the MED site (Fig. 1) did not show any phosphatase activity. Moreover, a substitution of Gln for the putative phosphatase binding residue R18 (Fig. 1) resulted in a reduction in the K_m by 3.5-fold and k_{cat} by 2.7-fold. These data suggested that the substituted residues, ²¹MED²³, ¹⁰⁵DGH¹⁰⁷ and R18, build the active center and are essential for PPM1D phosphatase activity.

Most PPM1D substrates often contain acidic residues in the regions flanking the dephosphorylated recognition sites [5, 6, 7]. To confirm the possible effects of acidic amino acids in the substrate affinity for PPM1D, a series of WT peptide analogs with different numbers of charged residues was synthesized and the kinetic parameters of these products were analyzed. p53(10-23)15P-TK (TK), in which T18 of WT was replaced by the basic amino acid lysine, showed a 1.5-fold higher K_m than WT (Table 1).

Table 1. Kinetics parameters of the dephosphorylation of synthetic phosphopeptides by PPM1D

Substrate	Sequences ^a	K_m (μ M)	k_{cat} (S^{-1})
WT	Ac-VEPPLS(P)QETFSDLW-NH ₂	12.6 \pm 1.9	4.6 \pm 0.2
TK	Ac-VEPPLS(P)QEKFSDLW-NH ₂	N.D. ^b	N.D. ^b
3K	Ac-VKPPLS(P)QKTFSKLW-NH ₂	10.4 \pm 1.4	4.2 \pm 0.2
TD	Ac-VEPPLS(P)QEDFSDLW-NH ₂	17.7 \pm 2.6	3.5 \pm 0.2
3E	Ac-VEPPLS(P)QEEEEDLW-NH ₂	6.3 \pm 1.0	3.0 \pm 0.1

Values represent average from three independent experiments.

Phosphorated amino acids are indicated in bold. Acidic residues are in italic.

The K_m and k_{cat} values could not be determined due to the high K_m .

Furthermore, an acidic residue deficient analog, p53(10-23)15P-3K (3K) could not be dephosphorylated by PPM1Dc (Table 1). These data suggested that acidic residues in the flanking regions of the dephosphorylation site are important for substrate recognition by PPM1D.

To next evaluate the effects of substituting acidic residues into PPM1D substrates, kinetic analyses of the p53(10-23)15P-TD (TD) substrate were carried out. The K_m value for TD was slightly lower than the WT (Table 1). A more acidic substrate peptide p53(10-23)15P-3E (3E) showed the lowest K_m . It was noteworthy also that the 3E peptide showed a low k_{cat} value despite of its high affinity for PPM1D (Table 1). An explanation for this may be that the off-rate of 3E from its binding site in PPM1Dc is lower than WT, or may be that 3E can bind to PPM1Dc at a different site and inhibit the activity of PPM1D. These data thus suggested that 3E is a candidate PPM1D inhibitor.

To develop peptidic inhibitors for PPM1D the non-hydrolyzable phosphoserine mimetic, D,L-2-amino-4-phosphonobutyric acid (AP4), was introduced into the 3E peptide. Purification of crude AP4-3E peptide by HPLC showed two major peaks, AP4-3E-A and AP4-3E-B, considered as diastereomers. Inhibitory activity analyses revealed that both of AP4-3E peptides strongly inhibited PPM1D in a non-competitive manner (Fig.2). This is the first report of the PPM1D inhibitor that functions in a non-competitive manner. Non-competitive inhibitor is known to work as more effective inhibitor than competitive one in the situation with accumulated substrate and AP4-3E may be effective inhibitor for PPM1D.

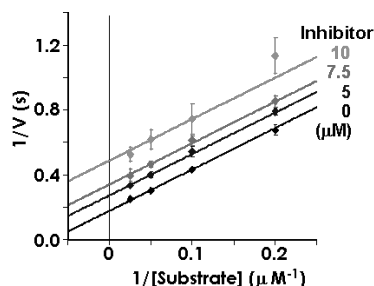


Fig. 2. Inhibition of PPM1D activity with the p53 peptide analog. Sequence of AP4-3E is Ac-VEPPL(AP4)QEEEEDLW-NH₂

In order to investigate the substrate recognition of PPM1D, homology modeling of PPM1D was carried out. The homology modeling showed that the putative metal

chelating residues and phosphate binding residue of PPM1D were located closely to each other and build the active center. Our kinetics analysis using PPM1D mutants and structure modeling support the appropriate alignment is necessary for proper modeling.

Homology modeling indicated that the B-loop is located on the same side as the catalytic center. This may account for the fact that acidic residues are often seen in the regions flanking the dephosphorylation sites in PPM1D substrates. Another PPM1D characteristic loop, the Pro-loop, is located on the opposite side of the catalytic center. Pro-rich sequences often play an important role in protein-protein interaction, suggesting that the Pro-loop in the PPM1D protein may play an important role in the interaction of this phosphatase with other proteins. Further studies will be required in the future to clarify the function of these characteristic loops in PPM1D. We have also developed a unique non-competitive inhibitor of PPM1D, the AP4-3E peptide. Such an inhibitor should offer advantages over its competitive counterparts in blocking this phosphatase due to the shallow substrate binding pocket in PPM1D. AP4-3E could therefore prove to be a useful lead compound in the development of potential therapeutic inhibitors of PPM1D with a high affinity and selectivity. The model based on precise sequence alignment should be useful for analysis of substrate recognition mechanism and for designing specific PPM1D inhibitors.

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