A trapped human PPM1A–phosphopeptide complex reveals structural features critical for regulation of PPM protein phosphatase activity

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Abstract

Metal-dependent protein phosphatases (PPM) are evolutionarily unrelated to other serine/threonine protein phosphatases and are characterized by their requirement for supplementation with millimolar concentrations of Mg2+ or Mn2+ ions for activity in vitro. The crystal structure of human PPM1A (also known as PP2Cα), the first PPM structure determined, displays two tightly bound Mn2+ ions in the active site and a small subdomain, termed the Flap, located adjacent to the active site. Some recent crystal structures of bacterial or plant PPM phosphatases have disclosed two tightly bound metal ions and an additional, third metal ion in the active site. Here, the crystal structure of the catalytic domain of human PPM1A, PPM1Acat, complexed with a cyclic phosphopeptide, c(MpSIpYVA), a cyclized variant of the activation loop of p38 MAPK (a physiological substrate of PPM1A), revealed three metal ions in the active site. The PPM1Acat D146E–c(MpSIpYVA) complex confirmed the presence of the anticipated third metal ion in the active site of metazoan PPM phosphatases. Biophysical and computational methods suggested that complex formation results in a slightly more compact solution conformation through reduced conformational flexibility of the Flap subdomain. We also observed that the position of the substrate in the active site allows solvent access to the labile third metal-binding site. Enzyme kinetics of PPM1Acat toward a phosphopeptide substrate supported a random-order, bi-substrate mechanism, with substantial interaction between the bound substrate and the labile metal ion. This work illuminates the structural and thermodynamic basis of an innate mechanism regulating the activity of PPM phosphatases.

Reversible protein phosphorylation signaling pathways are shaped by opposing actions of protein kinases and phosphatases. These pathways regulate the response of cells and organisms to changing environmental and physiological circumstances; dysregulation of these pathways underlies many human diseases. Although phosphorylated protein residues are dominated by phosphoserine (pSer)2 and phosphothreonine (pThr), with a minimal proportion of phosphotyrosine (pTyr), the numbers
of phosphatases involved in reversing the pSer/pThr and pTyr modifications are more evenly divided (1-3). Analysis of global dynamics of pSer/pThr and pTyr-based signaling suggests distinct patterns of regulation (4). The phosphoprotein phosphatases (PPP), with 13 members, and the metal-dependent phosphatases (PPM), with 18 members, provide most of the serine/threonine protein phosphatase activity in human cells (5,6). The activity, substrate specificity, and subcellular localization of PPP phosphatases are regulated primarily by binding of regulatory or inhibitor subunits, of which over 100 have been identified in human cells (7,8). PPM phosphatases generally are monomeric, but regulation of their activity remains incompletely understood (9-11). The evolutionarily distinct PPP and PPM phosphatases both feature tightly-bound bi-metal clusters in their active sites, but only the PPM phosphatases require supplementation with millimolar concentrations of Mg2+ or Mn2+ salts to exhibit detectable activity in vitro, suggesting that a weakly-bound ion is necessary for activity (5,6).

Human PPM1A (protein phosphatase, Mg2+/Mn2+ dependent 1A), also known as PP2Cα, was the first PPM phosphatase to be structurally characterized (12). PPM1A has diverse substrates and generally functions as a negative regulator of stress response pathways. PPM1A directly dephosphorylates multiple members of the mitogen-activated protein kinase (MAPK) signaling pathway, including ERK, JNK and p38 MAPK (13,14). PPM1A is a tumor suppressor due to this negative regulation of MAPK signaling pathways, with consequent activation of p53, as well as its specific dephosphorylation of cyclin dependent kinases (14-16). PPM1A also negatively regulates inflammatory and developmental processes through the TGFβ, nuclear factor kappa B (NFkB), and TNFα signaling pathways (17-19). Interestingly, PPM1A increases insulin sensitivity by positively regulating PI3K activity through dephosphorylation of the regulatory subunit PI3KR1 (20).

Existing structures of full-length, human PPM1A were determined from crystals formed at pH 5.0 to 5.5 and exhibit two closely-spaced Mn2+ ions in the active site (12,21). The PPM1A catalytic domain forms an αββα sandwich, with a peculiar structure termed the Flap positioned at one end of the active site; a C-terminal extension comprising helices α7 to α9 is specific to PPM1A/PPM1B homologues (12,22). The Flap structure has been proposed to increase specificity through interactions with substrates (23). The bi-metal center features an indirectly coordinated phosphate ion and a bridging water/hydroxide ion that is proposed to function as the nucleophile for the SN2 hydrolase reaction (12,21). As these Mn2+ ions have sub-micromolar affinities, their presence does not explain the requirement for supplementation with millimolar concentrations of Mg2+ or Mn2+ ions (5,6). An additional divalent ion, M3, which is displayed as a third ion present in the active sites of some crystal structures of bacterial or plant PPM phosphatases, has been proposed as the weakly-bound, catalytically essential metal ion (24-28). As most M3-coordinating residues are highly conserved, the involvement of M3 in metazoan PPM catalysis was anticipated (24,26-28). Recently, we used isothermal titration calorimetry (ITC) to detect millimolar-affinity binding of Mg2+ to PPM1A (29). Site-directed mutagenesis of D146 or D243 was used to distinguish two mutually exclusive subsites for the third metal ion: the D146/D239 subsite, which supports catalysis, and the D239/D243 subsite, which increases metal and substrate affinities but is not essential for catalysis (29). D239 coordinates M1 in addition to its involvement in both M3 subsites; D239 mutants are inactive. We used hydrogen-deuterium exchange-mass spectrometry (HDX-MS) to investigate the conformational mobility of PPM1A in the presence of low or moderate magnesium ion concentrations (30). We found that the binding of Mg2+ to the catalytically essential D146/D239 subsite restricted conformational mobility of the active site and specific regions of the Flap subdomain, suggesting that the third metal ion stabilizes the native structure of the Flap subdomain, suggesting the idea that conformational stabilization of the Flap, which is critical for specific substrate recognition, may be coupled to catalytic activity through the binding of the third metal ion.

Results

Substrate binding by inactive mutants of PPM1A

Substrates of PPM1A exhibit few commonalities in flanking sequences (Fig. 1A) (14,15,18,31). Apart from the prevalence of hydrophobic residues in the +1 position, the
targeted pSer/pThr residues are flanked by an assortment of charged, polar and hydrophobic residues without a unitary pattern. To gain insight into structural aspects of substrate recognition and the involvement of the third metal ion in human PPM1A catalytic activity, we sought conditions for co-crystallization of an inactive mutant of PPM1A in complex with a phosphopeptide substrate under physiological pH and in the presence of Mg\(^{2+}\) ions. Trapping of substrate phosphoproteins by inactive phosphatases has been used to isolate phosphatase-substrates complexes (29,32,33). PPM1A D146A is catalytically inactive (29,34), a defect that may result from reduced substrate binding and/or disruption of catalysis. Hence, we investigated the binding of fluorescein-labeled p38(175-185, 180pT) to PPM1A D146A by fluorescence anisotropy (Fig. 1B). The inactive mutant binds the labeled phosphopeptide with \(K_d = 48 \pm 16 \, \mu M\), thus demonstrating that the D146A mutation does not abolish substrate binding. Michaelis constants, determined from the substrate concentration dependence of the initial rates, provide an estimate of the strength of substrate binding. The \(K_m\) for the labeled phosphopeptide binding to PPM1A D146A is 1.8-fold weaker than the reported \(K_m\) of PPM1A for unlabeled phosphopeptide (27 ± 4 \, \mu M) and nearly equal to the corresponding \(K_m\) of PPM1A D243A (53 ± 9 \, \mu M) (29). Note that while PPM1A D243A is catalytically active, it exhibits reduced affinity for the catalytically-essential metal ion and a higher \(K_m\) for the substrate (29).

We further investigated the binding of the phosphopeptide to PPM1A D146A by NMR spectrometry chemical shift perturbations (Fig. 1C). TOCSY spectra of p38(175-185, pT180) alone (blue contours) or in the presence of full-length PPM1A D146A (10:1 molar ratio, red contours) showed significant chemical shift changes of the pT180 and G181 peaks, consistent with conformational changes of the modified and adjacent residues. A screen for crystallization conditions for the PPM1A D146A – p38(175-185, 180pT) complex failed to produce co-crystals.

The recombinant PPM1A protein used for these experiments retained four linker residues (SGGT) and the position 1 methionine following removal of the N-terminal 6X-His tag (29). As the initiating methionine is absent in mature, endogenous PPM1A and existing crystal structures begin with Gly2, the additional amino acids may interfere with crystallization. Moreover, HDX-MS experiments suggested increased conformational mobility of PPM1A D146A compared with the WT enzyme (30). To address these concerns, we reverted the N-terminus to the endogenous form, introduced the more conservative Asp to Glu mutation at position 146, and removed helices \(\alpha7, \alpha8, \) and \(\alpha9\) to produce the WT PPM1A\(_{cat}\) and PPM1A\(_{cat}\) D146E variants of the catalytic domain (aa 2–297). As expected, PPM1A\(_{cat}\) exhibited nearly the same activity as had been reported for His tag-free, full-length PPM1A, both for a phosphopeptide substrate, p38(175–185, 180pT), and a non-physiological substrate, \(\text{p-nitrophenyl phosphate (pNPP)}\) (Table 1) (29,34). PPM1A\(_{cat}\) D146E did not exhibit detectable activity for either substrate. The motif 5 aspartate residue (aligning to PPM1A D146) is highly conserved and intolerant of substitution, supporting its proposed role in coordinating a metal ion essential for catalysis (29,34-37).

We sought a tighter-binding substrate to increase the stability of potential protein-phosphopeptide complexes. Based on the p38\(\alpha\) activation loop sequence, the cyclic peptide \(\text{c(MpSlpYVA)}\) (Fig. 1D) had been developed as a substrate-competitive inhibitor of the human PPM parologue PPM1D; interestingly, it is a substrate of PPM1A, with a \(K_m\) of 11 \, \mu M (38). The shorter enzyme, PPM1A\(_{cat}\), also was active toward the cyclic peptide with a \(K_m\) of 5.9 ± 1.4 \, \mu M, while PPM1A\(_{cat}\) D146E did not exhibit measurable activity (Table 1). We used ITC to analyze the binding of \(\text{c(MpSlpYVA)}\) to PPM1A\(_{cat}\) D146E (Fig. 1E). After subtracting heats of dilution, integrated injection heats were fitted to a single site binding model, yielding the thermodynamic constants, \(\Delta H = -4.83 \pm 0.18 \, \text{kcal mol}^{-1}\) and \(\Delta S = 14.3 \, \text{eu}, \text{with} n = 0.906 \pm 0.031\). The enthalpic term contributes 70\% of the free energy of binding. The dissociation constant of the PPM1A\(_{cat}\) D146E – \(\text{c(MpSlpYVA)}\) complex, \(K_d = 8.9 \pm 0.3 \, \mu M\), is 1.6-fold weaker than the enzymatic \(K_m\) of PPM1A\(_{cat}\) for the cyclic peptide.

**Crystal structure of PPM1A\(_{cat}\) D146E in complex with the cyclic peptide c(MpSlpYVA)**

High quality monoclinic crystals of the PPM1A\(_{cat}\) D146E–c(MpSlpYVA) complex were obtained from a pH 7.3 solution containing Ca\(^{2+}\) ions (Table 2). Refinement of the structure to 2.2 Å...
resolution revealed an asymmetric unit comprising three similar protein-peptide complexes related by two pseudo-rotation axes. Consistent with structures of full length PPM1A (12,21), the truncated protein forms the characteristic PPM fold with the metal ion-containing active site embedded in the top edge of the opposed β sheets flanked by α helices (Fig. 2A). The Flap subdomain is positioned alongside the active site. The mutated residue, D146E, is located at the apex of the β6–β7 loop. The three complexes in the asymmetric unit exhibit moderate conformational variability, especially in flexible regions of the protein that exhibit high crystallographic B values (Supporting Information Fig. S1A, B).

Importantly, the active site contains three Ca$^{2+}$ ions and the bound cyclic peptide substrate. Two of the metal ions occupy positions corresponding to those of the two Mn$^{2+}$ ions found in structures of phosphate-bound, full length PPM1A obtained at pH 5.0–5.5 (12,21). Specifically, M1 is coordinated by D60, D239 and D282, with additional waters of coordination, and M2 is coordinated by D60, G61, and one of the phosphate oxygens of the cyclic peptide phosphoserine residue, with additional waters of coordination (Fig. 2B). Importantly, the bridging water/hydroxide, which has been proposed to serve as the nucleophile for the $S_n2$ reaction, is jointly coordinated by M1 and M2, as in the structures of full length PPM1A (12,21). The third metal ion is bound between the side chains of D239 and D243, one of the two proposed subsites for M3 (29). Based on the effects of specific mutations, the M3 D239/D243 subsite contributes to substrate affinity while the M3 D146/D239 subsite is essential for catalytic activity (29). The cyclic peptide sits in the active site with the phosphoserine positioned asymmetrically over the M1-M2 cluster and with the phosphotyrosine side chain proximal to the PPM1A N terminal residue, G2 (Fig. 2B). The three phosphoserine phosphate oxygens each can form a hydrogen bond with a M1 or M2-bound water molecule, supporting the importance of the M1/M2 cluster for charge stabilization of the transition state (12). As an $S_n2$ reaction, the phosphomonoester hydrolysis reaction proceeds with inversion at the phosphorous atom (6,39). Importantly, the position of the incipient leaving group, pSer$_{\gamma}$ O$_{\gamma}$, opposite to the M1/M2 bridging water molecule/hydroxide ion provides structural support for the role of the latter as the $S_n2$ nucleophile. Additional hydrogen bonds between the R186 side chain and the cyclic peptide Met$_1$ and pSer$_2$ backbone carbonyl groups stabilize the complex. Unlike the compact solution conformation of unbound c(MpSlpYVA) (38), the bound peptide exhibits an open conformation stabilized by intra-backbone hydrogen bonds (Supporting Information Fig. S1C). Except for the C₈ and O$_{\varepsilon}$ atoms of the mutated D146E residue, positions of the metal ions with coordinating waters, the cyclic peptide, and active site residues are well defined by localized electron density (Fig. 2C). The loss of defined conformation for the mutated side chain is consistent with the importance of the D146 residue for activity. Although mutation of Asp to Glu is regarded as a conservative substitution, in the context of the PPM active site, the additional methylene group displaces the O$_{\varepsilon}$ atoms from the proper distance to allow coordination of a divalent metal ion jointly with a D239 carboxylate oxygen. Representation of Coulombic potential in the active site shows the three divalent ions embedded in the highly negative field produced by active site aspartic acid δO atoms and surmounted by the positively-charged side chains of K165, R186, and R281 (Fig. 2D). Notable hydrophobic contacts between the protein and cyclic peptide are found between Flap residues I184 and L191 with cyclic peptide residues Val$_5$ and Ile$_3$, respectively, and between PPM1A residues A3 and F4 with cyclic peptide residues pTy$_{r4}$ and Met$_1$, respectively (Fig. 2E).

Calcium ions inhibit PPM1A activity, although the mechanism of inhibition has not been established (29,40). Coordination geometries of the metal ions are altered in the calcium-containing PPM1A$_{cat}$ D146E–c(MpSlpYVA) complex (Supporting Information Fig. S1D). In existing structures of full length PPM1A, both Mn$^{2+}$ ions exhibit octahedral coordination (12,21). For all three complexes in the asymmetric unit, M2 exhibits octahedral geometry, but the sixth coordination position is occupied by a phosphate oxygen of the cyclic peptide phosphoserine. The best-fitting coordination geometry for M1 varies among the three complexes in the asymmetric unit. M1 is coordinated by side chain carbonyl oxygens of D60, D239 and D282, with three or four water molecules occupying the remaining positions for trigonal prism, square face monocapped (chain A, $n = 7$, $rsmd = 0.312$), pentagonal bipyramid (chain
Comparison of phosphate-bound and cyclic peptide-bound forms of the PPM1A catalytic domain

The structures of the PPM1Acat D146E–c(MpSIpYVA) complex and the catalytic domain portion (aa 2–297) of phosphate-bound, full-length (aa 2–382) PPM1A are similar, with substantial conformational differences evident for the N-termini, the β3–β4 loops, the α2–β5 bridge structures, and the protruding points of the Flap (Fig. 3A). Within the active site, smaller conformational changes reflect specific interactions with the substrate (Fig. 3B). A shift in the position of the N-terminus permits hydrogen bonding between the N-terminal hydrogens and the phosphotyrosine Oη oxygen. In the full-length protein, the side chain of R33 protrudes into the active site, allowing hydrogen bonding or electrostatic interactions between both Nε hydrogens and two of the phosphate oxygens (12). In the complex, the R33 side chain is retained at the edge of the active site by a hydrogen bond between Nε(H) and the protein A3 backbone carbonyl oxygen. The side chains of Flap residues I184 and Q185 are shifted approximately 3 Å toward the bound substrate, compared with their positions in the phosphate-bound, full-length protein. In the complex, the Nε1 and Nε2 of R186 form hydrogen bonds with the cyclic peptide Met1 and pSer2 backbone carbonyl oxygens.

Comparative studies of protein sequences among homologues may allow discrimination between regions of the protein that are functionally constrained from those that are conformationally permissive. We compared the locations of conformational differences between the structures of the free protein and the cyclic peptide-protein complex with locations of sequence variability or insertions and deletions among 150 PPM1A homologues (Fig. 3C). Interestingly, the N-terminal amino acids and the Flap domain, which showed substantial differences in conformation between the free and bound protein, exhibit low sequence variability, suggesting the conformational differences may have functional significance. The α2–β5 bridge, also located on the top of the protein, exhibited substantial differences in conformation but exhibited a moderate level of sequence variability. The β3–β4 and α1a–α2 loops, located on the bottom of the protein, exhibited substantial differences in conformation and large variation in sequence, suggesting tolerance for structural variation. Insertions or deletions were tolerated only in the α2–β5 bridge and the β3–β4 and α1a–α2 loops, regions with high to moderate sequence variation. The β5–β6 and β7–β8 loops, also located on the bottom of the protein, exhibited substantial sequence variation but did not show conformational changes between the phosphopeptide-bound and phosphate-bound forms. The structural comparison of the free and substrate-bound forms of the PPM1A catalytic domain and the analysis of sequence variation among metazoan homologues support the importance of the first few N-terminal residues, the α2–β5 bridge, and the Flap subdomain in interactions between the enzyme and substrate. Recently, crystal structures of the Arabidopsis thaliana PP2C phosphatase TAP38/PPH1 in the free state and as the D180E mutant in complex with a phosphothreonine-peptide substrate were described (37). Similar to conformational changes of PPM1A residues R33 and R186 described above, the conformations of the TAP38/PPH1 residues R69 and R225 are altered upon binding of the substrate (37).

Small-angle X-ray Scattering and Molecular Dynamics

Recently, we used HDX–MS to characterize the conformational mobility of full-length PPM1A and PPM1A D146A under conditions of low (0.1 mM) and moderate (2 mM) Mg\(^{2+}\) concentrations (30). The Flap subdomain was identified as a conformationally mobile region in which amide hydrogen-deuterium exchange was affected both by Mg\(^{2+}\) concentrations and the identity of the residue at position 146. The Flap subdomain of bacterial PP2C phosphatases had been characterized as flexible based on its variable conformations in
crystals where its conformation may be affected by interactions with neighboring molecules (26-28). To gain additional understanding of the effects of the cyclic peptide binding on solution conformations of PPM1A, we used small angle X-ray scattering (SAXS) and molecular dynamics (MD) simulations. SAXS scattering profiles for PPM1Acat and PPM1Acat D146E are indistinguishable (Supporting Information Fig. S2A), indicating that the D146E mutation does not induce detectable changes in the solution conformation of the phosphatase under these conditions. Analysis of scattering profiles of the free and cyclic peptide-bound forms of PPM1Acat D146E (Fig. 4A, cyan and pink dots, respectively, and Supporting Information Fig. S2B) indicates that the radius of gyration of the complex (Rg, yr = 21.07 ± 0.06 Å) is slightly reduced compared with that of the free protein (Rg, yr = 21.51 ± 0.06 Å). Scattering profiles calculated from the crystal structures of phosphate-bound (residues 2-297 of PDB: 1A6Q) PPM1A and cyclic peptide-bound (present work, chains A, D) PPM1Acat D146E produced accurate fits to the scattering data (χ = 1.18 for the free protein and χ = 1.53 for the complex) (Supporting Information Fig. S2C). We used constrained MD simulations to generate conformational ensembles for free and cyclic peptide-bound PPM1Acat D146E proteins. Scattering profiles predicted from the constrained MD conformational ensembles provided slight improvements of the fits to the scattering data for the free protein and the complex (Fig. 4A, blue (χ = 1.12) and red (χ = 1.47) curves, respectively). The MD simulations also suggest that the Flap subdomain is more flexible in the free protein than in the protein-cyclic peptide complex. Analysis of conformational variation across the ensembles for the free protein (blue curve) and the protein-cyclic peptide complex (red curve) identifies regions of differential variability (Fig. 4B). Specifically, the MD simulations indicate reduced conformational variability in the α2-β5 bridge and the Flap subdomain in the complex, compared with the free protein. The reduction in Flap subdomain conformational variability upon complex formation is consistent with the observed small reduction in Rg, yr upon complex formation. To gain additional insight into the effects of cyclic peptide binding and the D146E mutation on conformational variability, we also performed MD simulations on the WT protein, without and with bound cyclic peptide, and compared these to the corresponding states of the D146E protein. Overlays of snapshots spanning the simulation interval for the WT protein, in free and cyclic peptide-bound forms (Supporting Information Fig. S3A and S3B, respectively) and the D146E mutant protein, in free and cyclic peptide-bound forms (Supporting Information Fig. S3C and S3D, respectively) illustrate conformational differences. In these simulations, M3 is bound to the D146/D239 subsite for the WT enzyme and to the D239/D243 subsite for the D146E mutant (Fig. 4C). In all four states, the weakly bound M3 ion exhibits greater positional variation than the tightly bound M1 and M2 ions (Fig. 4C). Among the four states, the positional variation of M3 is the largest for the WT enzyme in the unbound state (Fig. 4C, upper left). Conversely, the R33, H62 and R186 side chains exhibit the largest conformational variation for the D146E protein in the unbound state (Fig. 4C, lower left). Interestingly, for both the WT enzyme and the D146E protein, the R33, H62 and R186 side chains exhibit reduced conformational variability in the cyclic peptide-bound state compared with the free state (Fig. 4C, upper right and lower right panels, respectively). To provide a quantitative measure of the conformational variability of the Flap, we analyzed the distributions for the M1 to R186 Cα distance and the M2 to R186 Cα distance for the four MD conditions (Fig. 4D, upper and lower panels, respectively). As noted above, the positions of the tightly bound M1 and M2 ions show little variation across the simulation and provide relatively fixed reference points in the active site, whereas the Cα of R186 serves as an indicator of the position of the Flap. For both the WT and D146E proteins, the free protein exhibited considerably greater conformational variation compared with the respective bound states. The distributions for the free WT protein (blue curves) contain two peaks and are broader than those of the D146E protein (green curves). The distance distribution for the PPM1Acat-cyclic peptide complex (orange curve) is similar to that of the PPM1Acat D146E-cyclic peptide complex (red curve), but additionally contains a small peak with substantially larger distances. In the minor population, the R186-cyclic peptide hydrogen bond is disrupted, and the Flap is positioned farther from the active site. Mutation of PPM1A D146 to either alanine or glutamic acid abolishes catalytic
activity, implying that divalent ion occupancy of the M3 D146/D239 subsite is essential for catalytic activity. The simulations suggest that the binding of M3 to the D146/D239 subsite supports a conformationally restricted substrate-binding conformation, but also supports a minor population featuring a more distant flap conformation.

The catalytically-essential metal ion and the substrate bind in either order

An important aspect of the PPM1Acat D146E–c(MpSlpYVA) complex structure is the combined presence of a substrate and a third metal ion in the active site. This structure provides a context for investigating the interactions between binding of the substrate and binding of the third metal ion. A surface representation of the complex suggests that M3 bound in the D239/D243 subsite is accessible to the solvent, implying that the weakly bound ion can dissociate and re-associate in the presence of bound substrate (Fig. 5A). Moreover, the structure suggests that the catalytically essential D146/D239 subsite also is accessible to mobile ions. These observations suggest that the random-order, bi-substrate mechanism, in which the substrate (S) and the essential metal ion (M) may bind in either order, is the appropriate model (Fig. 5B). Note that for the purposes of kinetic mechanism, the essential metal ion refers to the weakly-bound ion provided by supplementation; the M1 and M2 ions, which also are necessary for catalysis but remain fully bound under normal conditions, are formally considered as intrinsic components of the free enzyme. The ordered sequential mechanism, an alternative mechanism, was proposed on the basis of product inhibition studies (40). In the ordered sequential mechanism, the obligatory initial binding of the metal ion is followed by binding of the substrate to form the catalytically competent ternary complex; after the catalytic step, the alcohol, phosphate ion, and metal ion are released sequentially (40). Evidence against the sequential mechanism is provided by the structure of a complex of an A. thaliana PP2C phosphatase with a trapped phosphopeptide substrate that contains two metal ions in the active site (37) and by an extensive mutagenesis study of a bacterial PP2C phosphatase that demonstrated the requirement for an intact M1-M2 bimetal cluster for efficient substrate binding (33). To investigate interactions between binding of the substrate and binding of the third metal ion, we determined the substrate- and magnesium ion-concentration dependencies of the initial rates for wildtype PPM1Acat and for PPM1Acat D243A, which retains only the D146/D239 subsite for M3. The substrate and magnesium ion concentration dependency of the initial rates each follow apparent Michaelis-Menten kinetics (Fig. 5C, Supporting Information Fig. S4). Analysis of the functional dependence of the apparent Michaelis constants on Mg2+ or substrate concentration, as appropriate, allows estimation of the kinetic constants for the random-order bi-substrate mechanism (Fig. 5D, Table 3). The values of $k_{cat}$ estimated by sequential fitting to the paired equations [2] and [3] or [5] and [6] agreed to better than 1%. The D243A mutation had little effect on the catalytic rate constant, in agreement with previous work (29). For both the WT and the D243A mutant enzyme, this analysis suggests substantial interaction between the binding of the weakly-bound metal ion and the binding of the phosphopeptide substrate, as indicated by the small value of $\alpha$ (0.07–0.08). This analysis assumes that binding events involving the substrate and metal ion are in rapid equilibrium in the presence of a rate-determining catalytic step. The rapid equilibrium assumption implies that the product of equilibrium constants for the formation of the ternary EMS complex via initial binding of the metal ion ($K_1; K_2$) must equal the product of equilibrium constants for its formation via initial binding of the substrate (($K_3/\alpha$)($aK_1$)) (Fig. 5B, upper and lower paths, respectively). Hence, the affinities of the phosphopeptide for the free enzymes ($K_3/\alpha$) are weaker than the affinities for enzymes with M3 bound ($K_2$) by about a factor of 12. Similarly, the affinities of the essential divalent ion for the free enzymes ($K_1$) are weaker than the affinities for the enzyme-phosphopeptide substrate complexes ($aK_1$) by about a factor of 12. Moreover, the values of $\alpha$ for the WT and D243A mutant enzymes are similar. Interestingly, published data describing the Mn2+- and pNPP-concentration dependences of PPM1A phosphatase activity (40), when subjected to the same analysis, support the random-order, bi-substrate mechanism with $\alpha = 0.4$ (Supporting Information Fig. S5). Hence, the coupling between the binding of Mg2+ and that of a phosphopeptide substrate is five times stronger than the coupling between the binding of Mn2+ and that of the artificial substrate pNPP.
Discussion

In this work, we have presented the structure of a trapped cyclic phosphopeptide substrate complexed with the catalytic domain of PPM1A. The enzyme is inactive due to the presence of Ca$^{2+}$ ions and the D146E mutation, either of which abolishes activity and enables trapping of the substrate (29,40). The cyclic phosphopeptide substrate, c(MpSlpYVA), is a tighter-binding variant of the p38 MAPK activation loop, a physiological substrate of PPM1A (14,38). Although the binding of millimolar-affinity Mg$^{2+}$ to PPM1A was detected by ITC (29) and the presence of the third metal ion in the active site was anticipated based on homology (24,26-28), the PPM1A(2-297) D146E-c(MpSlpYVA) complex provides the first structural confirmation of a third metal ion in the active site of a metazoan PPM phosphatase. In the complex, the cyclic peptide serine phospho-ester moiety is positioned asymmetrically over the M1–M2 cluster in the active site, with altered conformations of key PPM1A residues R33 and R186. The binding of c(MpSlpYVA) to PPM1Acat D146E is dominated by the favorable enthalpy of complex formation, consistent with small conformational adjustments in the complex compared with the free enzyme. Analysis by a combination of SAXS and MD simulations of the free and cyclic peptide-bound forms of PPM1Acat D146E revealed a slightly more compact solution conformation upon complex formation, primarily involving reduced conformational flexibility of the Flap subdomain and supporting the importance of the Flap for substrate recognition.

Hydrolases frequently feature metal ions in their active sites (41). For PPM phosphatases, the closely-spaced M1–M2 cluster provides a highly specific binding environment for phosphate ions, pSer- and pThr-peptides, and phosphoprotein substrates (6,12,21). The requirements for specific binding of phosphoproteins are distinct from requirements for catalytic activity, as specific binding of substrates was observed by catalytically inactive mutants that preserve the M1–M2 cluster or in the presence of Ca$^{2+}$ ions, as reported previously (29,33,37) and in the present work. The M1 and M2 ions jointly coordinate a bridging water molecule/hydroxide ion that has been proposed to function as the nucleophile in the $S_{2}$2 phosphate monoester hydrolase reaction (6,12,21). In the original crystal structure of PPM1A, the position of the bound phosphate failed to indicate unambiguously which phosphate oxygen should model the serine/threonine leaving group, and thus the identification of the $S_{2}$2 nucleophile remained tentative (12). In the complex, the position of the phosphoserine Oγ, as the insipient leaving group, opposite to the bridging water molecule/hydroxide ion provides structural support for its identification as the $S_{2}$2 nucleophile. The M1-M2 cluster also may contribute to catalytic activity by reducing charge accumulation in the transition state (39), a function supported by the direct coordination of serine/threonine phospho-monoester oxygen atoms by M2, as seen in the A. thaliana PHH1-trapped substrate complex (37) and in the present work. Thus, the M1–M2 cluster is necessary for substrate binding, activates the water molecule/hydroxide ion to serve as the $S_{2}$2 nucleophile, and stabilizes the transition state through coordinated water molecules.

The role of the weakly-bound M3 in the PPM catalytic mechanism remains to be demonstrated. The absence of detectable enzymatic activity without supplementation with millimolar concentrations of divalent Mg$^{2+}$ or Mn$^{2+}$ is one of the defining characteristics of the PPM phosphatases (7,35). In eukaryotic and eukaryotic-like bacterial PPM phosphatases, detection of a third metal ion in the active site is correlated with the presence of the sequence GDS in motif 5, corresponding to aa 145-147 in PPM1A (6,35,36); mutation of the motif 5 aspartate residue in these phosphatases results in loss of activity (24,26-29,37). The location of the D146/D239 subsite suggests that M3 is needed to facilitate protonation of the serine/threonine leaving group by positioning an activated, metal-bound water molecule near the fission phosphate monooester bond (6,37). This role is supported by the location of the cyclic peptide phosphoserine Oβ–Oγ bond in the structure of the complex near the presumed D146/D243 M3 subsite. In addition, this role is supported by the correlated reductions in catalytic activity and apparent metal binding affinity ($K_{metal}$) with decreasing pH (29).

Divalent calcium ions were found to inhibit the activity of PPM1A toward pNPP as a competitive inhibitor of Mn$^{2+}$ with a $K_{i} = 4.45$ mM (40), suggesting that catalytic inhibition by Ca$^{2+}$ resulted...
from its occupancy of the weak M3 binding site. As the Lewis acidity of Mg$^{2+}$, Mn$^{2+}$ and Ca$^{2+}$ are similar, the failure of calcium to support catalysis may result from its increased size or its propensity to adopt alternative coordination geometries (42,43). Note that in the intracellular environment, competitive inhibition of PPM phosphatase activity by Ca$^{2+}$ ions is considered inconsequential because intracellular calcium ion concentrations are orders of magnitude lower than the $K_i$.

The dependence of the initial rates on phosphopeptide substrate and Mg$^{2+}$ concentrations supports a random-order, bi-substrate mechanism, with substantial interaction between the binding of the substrate and the labile metal ion. This mechanism is supported by the structure of the complex, which indicates that both the D146/D239 and D239/D243 subsites are accessible to ions in solution in the presence of bound substrate, and by solution studies suggesting that the binding of either Mg$^{2+}$ or substrate results in reduced conformational mobility (30). Although the substrate can bind in the absence of the weakly-bound metal ion, the binding affinity is considerably higher for the metal-bound state. Similarly, although Mg$^{2+}$ can bind to the substrate-free enzyme, the binding affinity is considerably higher for the substrate-bound state. As only the ternary complex is catalytically active, this mechanism suggests that the activities of PPM phosphatases can be regulated by the concentrations of both substrate and metal ions to effect negative regulation of signaling pathways. In most eukaryotic cells, the concentration of free Mg$^{2+}$ is less than 1 mM, with extensive competition among proteins, nucleic acids and lipids for binding (44). In the absence of stress signaling, low concentrations of phosphoprotein substrate result in limited formation of the ternary EMS complex. Following activation of a stress signaling pathway, the increased abundance of phosphorylated signaling protein drives formation of the active EMS ternary complex due, in part, to the increased affinity for Mg$^{2+}$. In addition, stress signaling may affect intracellular metal ion concentrations through altered ion channel activity, with consequent activation of PPM phosphatase activity (45-47). The resulting high activity of the phosphatase efficiently dephosphorylates the signaling protein, resulting in decommissioning of activated stress signaling.

### Experimental Procedures

#### Expression and purification of His tag-free PPM1A D146A

Expression and purification of His-TEV-PP2Ca D146A was performed as described previously, including treatment with tobacco etch virus (TEV) protease to remove the N-terminal His tag (29). The protein was further purified by anion exchange and gel filtration chromatography. For anion exchange chromatography, the protein was dialyzed against Buffer B (50 mM HEPES (pH 7.5), 2 mM β-mercaptoethanol, 2 mM MgCl$_2$, 0.1 mM EGTA and 10% glycerol) containing 100 mM NaCl. The protein was then loaded onto a Q-Sepharose column (GE-Healthcare) that was pre-equilibrated with Buffer B containing 100 mM NaCl. The column was subsequently washed with the same buffer, and the protein was eluted by using a 100–400 mM NaCl gradient in the same buffer. The pooled fractions from Q-Sepharose were concentrated. Gel filtration was performed by FPLC using Superdex-75 16/60 (GE-Healthcare) in the presence of Buffer B containing 100 mM NaCl, resulting in purified, full-length PPM1A D146A. Note that this protein retained five additional amino acids (SGGTM) at the N-terminus, compared with endogenous PPM1A. The protein was >95% pure as determined by SDS-PAGE followed by Coomassie Blue staining. Protein concentrations were determined by optical spectrometry (Nanodrop) using $e_{280} = 35410$ M$^{-1}$ cm$^{-1}$.

#### PPM1ACat PPM1ACat D146E and PPM1ACat D243A cloning, expression and purification

PPM1ACat (aa 2-297) cDNA was cloned into the Bsal-XbaI sites of pE-SUMOstar (LifeSensors) using PCR with the following oligonucleotide primers:

| Forward Oligonucleotide (5′ to 3′) | Reverse Oligonucleotide (5′ to 3′) |
|-----------------------------------|-----------------------------------|
| 5′-CTATGGTCTCAAGTGGAGCATTTTTAGACAACAGCCA-3′ | 5′-GGCCCTCTAGATTATACTTTGGGTGCATTTGGAAAACA-3′ |
| 5′-TGTTATCTGGGCTGTTATGGGAAATG-3′ | 5′-TCACATGCAAGGATAATGAAC-3′ |

The pooled fractions from Q-Sepharose were concentrated. Gel filtration was performed by FPLC using Superdex-75 16/60 (GE-Healthcare) in the presence of Buffer B containing 100 mM NaCl, resulting in purified, full-length PPM1A D146A. Note that this protein retained five additional amino acids (SGGTM) at the N-terminus, compared with endogenous PPM1A. The protein was >95% pure as determined by SDS-PAGE followed by Coomassie Blue staining. Protein concentrations were determined by optical spectrometry (Nanodrop) using $e_{280} = 35410$ M$^{-1}$ cm$^{-1}$. 

#### Directed Mutagenesis

D146A and D243A mutants were performed using Q5 Site-Directed Mutagenesis Kit (New England Biolabs) with the primer pairs:

- 5′-TAACTGTGGAGGAGTCAGAGTTACACAC-3′ and 5′-ATGAAATAAGTATGTTGGGGAGGTTTAC-3′
- 5′-TGGTGATCTGGGCTTGATTGGGAATG-3′ and 5′-TCACATGCAAGGATAATGAAC-3′ (D243A)
SUMOstar plasmids encoding the proteins were transformed into *Escherichia coli* BL21(DE3) and the cells were grown in LB media containing 100 µg/ml ampicillin at 37 °C until the O.D.600 reached 0.5-0.6. Protein expression was induced by addition of 0.3 mM IPTG and 1 mM MnCl₂ and cells were incubated at 30 °C overnight before being harvested by centrifugation. Pellets were resuspended in Buffer A (50 mM HEPES (pH 7.5), 2 mM MgCl₂, 2 mM β-mercaptoethanol, 10% glycerol, 0.2% Triton X-100, 0.1 mM EGTA) containing 150 mM NaCl and supplemented with EDTA-free protease inhibitor cocktail (Roche). Cells were lysed using a French press, and the lysate was cleared by centrifugation (40,000 × g for 30 min at 4 °C). Proteins were purified by affinity chromatography using TALON (Clontech) metal affinity resin. The pooled fractions were dialyzed overnight at 4 °C against buffer B containing 50 mM NaCl. The His₆-SUMOstar tag was removed by incubation with SUMOstar protease 1 (LifeSensors) for 1 h at 30 °C followed by incubation with TALON metal affinity resin equilibrated with Buffer B containing 50 mM NaCl. The protein was further purified by anion exchange chromatography as described above. Pooled fractions from the Q-Sepharose column were concentrated and subjected to FPLC gel filtration using Superdex-75 16/60 in the presence of Buffer C (10 mM MES (pH 7.0), 150 mM NaCl, 2 mM TCEP, 2 mM MgCl₂) supplemented with 5% glycerol. The proteins were >95% pure as determined by SDS-PAGE followed by Coomassie Blue staining. Protein concentrations were determined by optical spectrometry (Nanodrop) using the extinction coefficient ε₂₈₀ = 25,440 M⁻¹ cm⁻¹.

**Peptide synthesis and purification**

All chemicals and amino acids used were of analytical grade. The peptide p38(175-185, 180pT) (NH₂-TDDEM-pT-GYVAT-COOH) and cyclic peptide [c(MpS-I-pY-V-A)] were synthesized by solid-phase peptide synthesis using 9-fluorenylmethoxycarbonyl (Fmoc)/tert-butyl chemistry on Wang resin and Rink Amide AM resin, respectively. Phosphorylated Threonine, Serine, and Tyrosine were incorporated as a Fmoc-Thr[PO(OBz)OH]-OH, Fmoc-Ser[PO(OBz)OH]-OH and Fmoc-Tyr[PO(OBz)OH]-OH, respectively. For final thioether cyclization of c(MpSlpYVA), the N-terminus of the peptide was chloraocetylated using chloroacetic anhydride and N,N-diisopropylcetylamine (DIEA), following established methods with minor modifications (38,48). For consistency in reporting structural information concerning macrocyclic peptides, the peptide-like unit containing the thioether bond is designated as the defined chemical component 48V (49,50). Cleavage of the peptides from the resin was achieved using a cocktail of Reagent K (TFA/Thioanisole/Water/Phenol/EDT:82.5:5:5:5:2.5 v/v) and 1% Triisopropylsilane (TIPS) for 3 hours at room temperature. After removal of the resin by filtration, the filtrate was concentrated by flushing with nitrogen gas. Crude peptides were precipitated with diethyl ether. For synthesis of c(MpSlpYVA), after cleavage from the resin, crude peptide was cyclized by dissolving in a 1% triethylamine-containing distilled, deionized water (Millipore) (~3 mM) and stirring at room temperature overnight. The cyclization reaction was quenched by acidification with acetic acid. Crude peptides were purified using reversed-phase high-performance liquid chromatography (RP-HPLC) on a preparative C4 column (BioAdvantage Pro 300, Thomson Liquid Chromatography) with 0.05% trifluoroacetic acid/water/acetonitrile as the solvent. Purified peptides were characterized by matrix-associated laser desorption ionization time-of-flight mass spectrometry (MALDI MicroMX, Waters). The purities of the peptides were found to be > 98% using analytical RP-HPLC.

**Peptide labeling**

The peptide p38(175-185, 180pT) was labeled with 5(6)-carboxyfluorescein (Novabiochem) on the solid phase. Dry resin-bound N-terminal deprotected peptide was reacted with an excess of 5(6)-carboxyfluorescein, HOBT/HBTU, and DIEA in N-Methyl-2-pyrrolidone (NMP) in the dark for 24 hours. The resin was washed thoroughly with 20% piperidine in NMP until the wash solution became colorless. Resin was then washed consecutively with NMP and diethyl ether five times and finally dried under an N₂ atmosphere. After labeling, peptides were cleaved as described above and purified by RP-HPLC, and masses were checked by MALDI MicroMX.

**Fluorescence anisotropy binding assay**
Fluorescence anisotropy measurements were performed at 25 °C using a Perkin Elmer LS55 Luminescence Spectrometer. The protein PPM1A D146A was dialyzed overnight against 20 mM Tris (pH 7.5), containing 100 mM NaCl, 2 mM β-mercaptoethanol, 30 mM MgCl₂, 0.1 mM EGTA and 10% glycerol. Titrations were carried out in the same buffer using an initial concentration of 5(6)-Carboxyfluorescein-labeled p38(175-185, 180pT) peptide of 1 µM. Fluorescence anisotropy was measured with excitation at 480 nm and emission at 520 nm using 5 nm bandwidths. After mixing with PPM1A D146A, solutions were incubated for 2 min before fluorescence anisotropy measurements were taken. Each titration point was measured twice and titrations were performed four times. The binding constant, \(K_d\), was estimated from nonlinear least-squares fitting (GraphPad Prism 7.01) of background-corrected, averaged anisotropy values, \(A\), to the equation
\[
A = A_f + (A_b - A_f) \cdot [P_t]/(K_d + [P_t])
\]
where \(A_f\) and \(A_b\) are the anisotropy of the free and bound peptide, respectively and \([P_t]\) is the dilution-corrected total protein concentration.

**NMR spectroscopy**

NMR experiments were conducted at 30 °C using a 600 MHz Bruker Biospin NMR spectrometer with a TCI cryoprobe. The protein PPM1A D146A and the peptide p38(175-185, 180pT) were co-dialyzed overnight against phosphate buffer (20 mM sodium phosphate (pH 7.5), 100 mM NaCl, 5 mM MgCl₂) and experiments were carried out in the same buffer. TOCSY (Total correlation spectroscopy; mixing time, 60 ms) and NOESY (nuclear Overhauser effect spectroscopy; mixing time, 400 ms) spectra were recorded in water suppression mode.

**Steady-state kinetics assays**

Phosphatase activity assays were performed as described previously (29). Under standard conditions, initial rates of phosphatase activity were determined by incubating proteins (15 ng, 9.2 nM) with various concentrations of phosphopeptide in phosphatase assay buffer (50 mM Tris-HCl (pH 7.5), 40 mM NaCl, 0.1 mM EGTA, 0.02% β-mercaptoethanol) supplemented with 30 mM MgCl₂ for 9 min at 30 °C. The dependence of initial rates on divalent metal ion concentration was determined as described above, except that the MgCl₂ concentrations were varied from 0.5 to 30 mM. Phosphate was determined using the Biomol green assay (Enzo Life Sciences), following the manufacturer’s protocol. Phosphatase activity toward p-nitrophenyl phosphate (pNPP) was measured using 120 ng (73.2 nM) protein in phosphatase assay buffer supplemented with 10 mM MnCl₂ for 12 min at 30 °C. The assay was terminated by adding 2 volumes of a 1:1 molar ratio of 2 N NaOH and 0.5 M EDTA (pH 8.0). The amount of p-nitrophenol released was determined spectrophotometrically (ε = 18,000 M⁻¹ cm⁻¹ at 410 nm). To determine \(K_m\) and \(k_{cat}\) values, the initial rates were fitted to the Michaelis–Menten equation using GraphPad Prism 7.01. Values of \(k_{cat}\) were calculated from the concentration of the enzyme, \([E]_o\) and the equation \(V_{max} = k_{cat} [E]_o\).

**Fitting initial rates to the rapid equilibrium, random-order, bi-substrate mechanism**

In the rapid equilibrium approximation, the initial rate of an enzymatic reaction that requires the binding of both substrate (S) and an essential divalent metal ion cofactor (M), in either order, is given by,
\[
v = \frac{V_{max} [M][S]}{K_1 K_2 + K_2 [M] + \alpha K_1 [S] + [M][S]} \quad [1]
\]
Equation [1] can be rearranged to take the form of a Michaelis-Menten equation with explicit dependence on the substrate concentration,
\[
v = \frac{V_{max}^{app(S)} [S]}{K_m^{app(S)} + [S]} \quad [2]
\]
wherein the dependence of the apparent Michaelis constants on [M] is given by
\[
V_{max}^{app(S)} = k_{cat} [M]/(\alpha K_1 + [M]) \quad [3]
\]
and
\[
K_m^{app(S)} = K_2 (K_1 + [M])/(\alpha K_1 + [M]) \quad [4]
\]
The parameter \(\alpha\) describes the degree of interaction between the binding of the substrate and the binding of the essential metal ion cofactor. Similarly, Equation [1] can be rearranged to take the form of a Michaelis-Menten equation with explicit dependence on the essential metal ion cofactor concentration,
\[ v = \frac{V_{\text{max}}^{\text{app}(M)} [M]}{K_{m}^{\text{app}(M)} + [M]} \]  

wherein the dependence of the apparent Michaelis constants on [S] is given by
\[ V_{\text{max}}^{\text{app}(M)} = k_{\text{cat}}[S]/(K_2 + [S]) \]
and
\[ K_{m}^{\text{app}(M)} = K_1 (K_2 + \alpha[S])/(K_2 + [S]). \]

Initial rates were determined as described above except that p38(175-185, 180pT) concentrations were varied from 20 to 200 μM for PPM1A_{cat} and from 30 to 300 μM for PPM1A_{cat} D243A; MgCl₂ concentrations were varied from 0.5 to 30 mM for the WT enzyme and from 1 to 30 mM for the D243A mutant. Initial rates were fitted by nonlinear least squares to equation [2] or equation [5] using Graphpad Prism. Nonlinear least squares methods were then applied to the dependence of \( V_{\text{max}}^{\text{app}(S)} \) on [MgCl₂] to estimate the parameters \( k_{\text{cat}} \) and \( \alpha K_1 \) (equation [3]) or applied to the dependence of \( V_{\text{max}}^{\text{app}(M)} \) on [S] to estimate the parameters \( k_{\text{cat}} \) and \( K_2 \) (equation [6]). Similarly, the parameters \( K_1 \) and \( K_2 \) were estimated from the dependence of \( K_{m}^{\text{app}(S)} \) on [MgCl₂] (equation [4]) with the value of \( \alpha K_1 \) held fixed at the determined value; the parameters \( K_1 \) and \( \alpha K_1 \) were estimated from the dependence of \( K_{m}^{\text{app}(M)} \) on [S] (equation [7]), with the values of \( K_2 \) held fixed at the determined value. The value of \( \alpha \) was determined either from the parameters of equations [3] and [4] or from the parameters of equation [7].

**Isothermal Titration Calorimetry (ITC)**

ITC measurements were performed at 25 °C using a VP-ITC MicroCalorimeter (MicroCal). PPM1A(2-297) D146E and the cyclic peptide c(MpSIpYVA) were individually co-dialyzed against Buffer C. ITC experiments were carried out in the same buffer with a protein concentration of 16 μM. Integrated heats of injection were corrected for the heats of dilution and were fitted to a one-site binding model using Origin 7.0 software.

**Crystallization and data collection and data processing**

PPM1A_{cat} D146E was purified as described above except that the buffer used for the Superdex 75 16/60 gel filtration, the buffer had the composition: 10 mM MES (pH 7.0), 150 mM NaCl, 2 mM TCEP and 2 mM MgCl₂. After concentration of protein to 20 mg/ml, the protein and cyclic peptide were co-dialyzed against the above buffer. The complex was formed by combining the protein and c(MpSIpYVA) in a 1:2 molar ratio and incubating at 25 °C for 1 h. In the crystallization trial, 3 μl of the complex was mixed with 3 μl of the well solution consisting of 0.1 M HEPES (pH 7.3), 0.1 M calcium acetate and 40% PEG400 in a hanging drop vapor diffusion experiment. Crystals grew over 3 days to an overall size of 200 × 200 μm at 20 °C and were cryoprotected by transfer into a solution prepared by diluting the complex buffer into 0.1 M HEPES (pH 7.3), 0.1 M calcium acetate, 40% PEG400 and 26% glycerol in 1:1 ratio for 25 min. Crystals were flash frozen directly in a nitrogen cryostream. Data were collected at 95 K, using a Rigaku 007HF rotating anode X-ray source producing CuKα radiation (1.5418 Å) and equipped with multilayer focusing mirrors on a Saturn A200 mosaic CCD detector in 0.25° oscillation images. The data were integrated and scaled internally using XDS and XSSCALE (51).

**Structure Solution and Refinement**

The crystal structure was determined by molecular replacement using AMoRe (52) with a model of PPM1A (PDB code 1A6Q) used in the search. Three molecules were found in the asymmetric unit. All subsequent calculations were carried out using CNS1.3 (53). The model was refined at 2.2 Å resolution with Cartesian simulated annealing, energy minimization and atomic displacement parameter optimization. Electron density was displayed and the model was manually modified where needed using O (54). Difference electron density for cyclic peptide c(MpSIpYVA) was apparent for all three molecules in the asymmetric unit. Chemical parameters restraining the cyclic peptide in crystallographic refinement were constructed so that they were consistent with the Engh and Huber protein restraint parameters (55) used throughout in the process. After further refinement, 10 Ca²⁺ ions with their associated coordinating waters and 606 additional ordered solvent water molecules were added. The final model was verified with a composite simulated annealed omit electron density map calculated in CNS 1.3. Data collection and refinement statistics
The Ramachandran plot of the final model indicated that 89.9% of all residues were in the most favored regions of the plot, while three residues (one in each monomer) were in the disallowed region.

Small-angle X-ray scattering

Before the experiments, the proteins and the c(MpSlpYVA) cyclic peptide were co-dialyzed against 10 mM MES, pH 7.0, containing 150 mM NaCl, 2 mM TCEP and 2 mM MgCl₂ and experiments were carried out in the same buffer. Solution X-ray scattering data were collected for samples containing between 3.0 and 0.5 mg/mL protein in order to investigate the effect of sample concentration on the scattering data. SAXS data collections were performed at beam line 12IDB at the Advanced Photon Source synchrotron (APS, Argonne National Laboratory, Argonne IL) or using a lab-based instrument (SAXSLAB, Institute for Bioscience and Biotechnology Research, Rockville MD), all carried out at 25 °C. Synchrotron data were collected using 14 keV incident radiation and Pilatus 2M detector covering the q-range between 0.008 Å⁻¹ and 0.96 Å⁻¹. Lab-based SAXS data were collected using 8 keV incident radiation from a Rigaku 007HF rotating anode source and a Pilatus 300K detector with programmable positioning covering the q-range from 0.008 Å⁻¹ to 0.8 Å⁻¹. Multiple data sets, including concentration series, were collected on both instruments yielding scattering profiles that were indistinguishable apart from data uncertainty. Scattering measurements reported here comprise 30 independent data frames acquired sequentially at APS with means calculated over the 30 frames and uncertainty in the mean calculated as the root-mean-square deviations over the 30 frames divided by the square root of 30. No data frames were removed. Two-dimensional scattering data were processed using instrument-specific routines correcting for the sample transmission, detector pixel sensitivity, and solid angle per pixel while using dynamic masking to remove the data impacted by stray cosmic radiation. Buffer scattering was subtracted from sample curves using protein concentration-based solvent volume fractions. The radii of gyration were determined using Guinier fits within the q-ranges corresponding to qmax·Rgyr < 1.1-1.2 using Primus software (56) while ensuring the absence of the systematic biases in the fit residuals. All fits of the scattering data to the atomic coordinates of the proteins were performed using AXES software (57), averaging over multiple ensemble members where appropriate.

Molecular Dynamics Simulations

All simulations were done with substitution of Mg²⁺ ions for the Ca²⁺ ions in the crystal structure. Four different states of the PPM1Acat protein were investigated: (1) the wildtype protein with a third Mg²⁺ ion occupying the D146/D239 subsite; (2) as in state (1) but with bound cyclic peptide; (3) the D146E mutant protein with a third Mg²⁺ ion occupying the D239/D243 subsite; (4) as in state (3) but with bound cyclic peptide. Trajectories were calculated with the NAMD 2.9 software (58) using the CHARMM36 All-Hydrogen Topologies and Parameters (59). Positional Harmonic Constraints were applied to the alpha-carbons of the core residues: residues 13-16, 24-27, 39-42, 56-60, 129-132, 140-143, 148-151, 159-162, 220-223, 234-237, 284-288, and 293-295. Proteins were centered in a cubic box with initial dimensions of 75.0 Å. The solvent was represented by the explicit TIPS3P model, and periodic boundary conditions were applied. A CUTOFF of 12.0 Å was used for the energy functions, with a SWITCHDIST of 10.0 Å and PAIRLISTDIST of 14.0 Å. The Langevin and Langevin-Piston algorithms were used to propagate the dynamics and maintain constant temperature and pressure of 300.0 K and 1 atm, respectively. Rigid bonds were used to allow for an integration timestep of 2.0 fs. Trajectories for each state were propagated for 300 ns, with the first 10 ns considered heating and equilibration and not included in the analysis.

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c(MpSIpYVA) complex are available in the Research Collaboratory for Structural Bioinformatics Protein Databank = PDB # 6B67. This work utilized the SAXS Core facility (CCR, NCI) and the computational resources of the NIH HPC Biowulf cluster, (http://hpc.nih.gov). SAXS data were collected at beamline 12-ID-B at the Advanced Photon Source (Argonne IL). The shared scattering beamline 12-ID-B resource is allocated under agreement PUP-24152 between the NCI and Argonne National Laboratory (ANL). Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility, was operated for the DOE Office of Science by ANL under Contract No. DE-AC02-06CH11357. We thank Dr. Lixin Fan (NCI), and Dr. Xiaobing Zuo (ANL) for expert support.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Footnotes

This article contains Supporting Information Figures S1–S5.

1These authors contributed equally to this work.

The abbreviations used are: DIEA, N,N-diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HDX-MS, hydrogen-deuterium exchange-mass spectrometry; ITC, isothermal titration calorimetry; MD, molecular dynamics; NFkB, nuclear factor kappa B; NMP, N-Methyl-2-pyrrolidone; NOESY, nuclear Overhauser effect spectroscopy; pNPP, para-nitrophenyl phosphate; PPM, metal-dependent protein phosphatases; PPP, phosphoprotein phosphatases; pSer, phosphoserine; pThr, phosphothreonine; pTyr, phosphotyrosine; RP-HPLC, reversed-phase high-performance liquid chromatography; rsmd, root-mean-square deviation; SAXS, small angle X-ray scattering; TIPS, Triisopropylsilane; TOCSY, total correlation spectroscopy.

3 The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. DISCLAIMER: Certain commercial materials and equipment were identified to adequately specify the experimental procedure. Such identification neither implies recommendation or endorsement by NIST nor does it imply that the material or equipment identified is the best available for the purpose.
| Substrate                  | $K_m$ (µM)$^2$ | $k_{cat}$, s$^{-1}$ |
|---------------------------|----------------|-------------------|
| PPM1A$^{cat}$             |                |                  |
| p38(175-185, 180pT)       | 40 ± 9         | 7.5 ± 0.7        |
| PPM1A$^{cat}$ D146E       | n.d.           | n.d.             |
| PPM1A$^3$                 |                |                  |
| p38(175-185, 180pT)       | 27 ± 4         | 6.3 ± 0.2        |
| PPM1A D146A$^3$           | n.d.           | n.d.             |
| PPM1A$^{cat}$             |                |                  |
| c(MpSIpYVA)               | 5.9 ± 1.4      | 4.5 ± 0.2        |
| PPM1A$^{cat}$ D146E       | n.d.           | n.d.             |
| PPM1A$^{cat}$             |                |                  |
| pNPP                      | 0.74± 0.1      | 2.3 ± 0.07       |
| PPM1A D146A$^3$           | n.d.           | n.d.             |
| PPM1A$^{cat}$             |                |                  |
| pNPP                      | 1.3 ± 0.1      | 2.9 ± 2          |

$^1$From initial rates determined at 30 °C in a buffer containing 50 mM Tris (pH 7.5), 40 mM NaCl, 0.1 mM EGTA, 0.02% β-mercaptoethanol and indicated divalent ions. n.d., not detected. $^2$In the presence of 30 mM MgCl$_2$. $^3$Previously published in Table 3 of Tanoue et al. (2013) Biochemistry 52, 5830-5843 as WT (PPM1A) and D146A (PPM1A D146A), each with additional residues SGGT at the N-terminus. Results are in the public domain and may be reproduced. $^4$In the presence of 10 mM MnCl$_2$. 

**Table 1. Kinetic constants for PPM1A variants**

- **PPM1A$^{cat}$**: Phosphopeptide complex.
- **Substrate**: Different phosphopeptides and pNPP.
- **$K_m$**: Michaelis constant (µM).
- **$k_{cat}$**: Catalytic constant (s$^{-1}$).
Table 2. Data collection and refinement statistics (molecular replacement)

| Data collection                              | PPM1A\textsubscript{cat} D146E–c(MpSIpYVA) complex\textsuperscript{1} |
|----------------------------------------------|---------------------------------------------------------------|
| **Space group**                              | C2                                                            |
| **Unit cell dimensions:**                    |                                                               |
| \(a, b, c\) (Å)                              | 159.03, 89.57, 70.05                                          |
| \(\alpha, \beta, \gamma\) (°)               | 90, 113.86, 90                                                |
| **Resolution (Å)**                           | 50-2.2 (2.26-2.20)\textsuperscript{2}                        |
| **\(R\text{measured}\)**                    | 3.6 (13.1)                                                    |
| \(I / \sigma I\)                             | 25.9 (4.63)                                                   |
| **Completeness (%)**                         | 95.7 (79.1)                                                   |
| **Redundancy**                               | 2.87 (1.25)                                                   |
| **Total observations**                       | 125802                                                        |
| **Unique reflections**                       | 43867                                                         |

**Molecular Replacement**

| MR model                                     | 1A6Q                                                          |
| **Correlation coeff.**                       | 16.5, 15.9, 15.0 (10.4 next highest noise peak)               |
| **R-factor (%)**                             | 38.0                                                          |
| **# monomers in a.s.u.**                     | 3                                                             |

**Refinement**

| **Resolution (Å)**                           | 30-2.2                                                        |
| **No. reflections (working set)**            | 42734                                                         |
| **\(R\text{work} / R\text{free}\)**         | 16.9 / 22.9                                                   |
| **No. atoms**                                |                                                               |
| Protein                                      | 6891                                                         |
| Ligand/ion                                   | 229                                                          |
| Water                                        | 606                                                          |
| **B-factors**                                |                                                               |
| Protein                                      | 23.66                                                         |
| Ligand/ion                                   | 43.48                                                         |
| Water                                        | 30.41                                                         |
| **R.m.s. deviations**                        |                                                               |
| Bond lengths (Å)                             | 0.012                                                         |
| Bond angles (°)                              | 4.0                                                           |

\textsuperscript{1}PDB # 6B67. \textsuperscript{2}Values in parentheses are for highest-resolution shell. \textsuperscript{3}Correlation coefficient (on F) of the solutions after rotation function; \textsuperscript{4}Crystallographic R factor of the solution after translation function and rigid body refinement; \textsuperscript{5}Computed using a randomly selected set of reflections representing 2.5% of the unique reflections.
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Table 3. Kinetic constants for random-order, bi-substrate mechanism

|                          | equation | PPM1A<sub>cat</sub> | PPM1A<sub>cat</sub> D243A |
|--------------------------|----------|----------------------|---------------------------|
| $k_{cat}$ (s<sup>-1</sup>)| [3]      | 4.2 ± 0.15,          | 4.4 ± 1.2,                |
|                          | [6]      | 4.2 ± 0.19           | 4.5 ± 0.3                 |
| $\alpha K_1$ (mM)       | [3]      | 0.41 ± 0.09          | 1.26 ± 0.32               |
|                          | [6]      | 16.7 ± 3.1           | 27.2 ± 7.8                |
| $K_2$ (μM)               | [4]      | 5.2 ± 0.5,           | 17.8 ± 3.4,               |
|                          | [7]      | 5.2 ± 2.9            | 14.8 ± 1.1                |
| $\alpha$                 | [3], [4] | 0.08,                | 0.07,                     |
|                          | [7]      | 0.08                 | 0.085                     |
| $K_2/\alpha$ (μM)        |          | 209                  | 350                       |

<sup>1</sup>Experiments were performed at 30 °C with 0-30 mM MgCl<sub>2</sub> and 0-300 μM p38α(175-185, 180pT). Initial rates were fitted to an apparent Michaelis-Menten equation with explicit dependence on Mg<sup>2+</sup> or substrate concentration.
Figure 1. Phosphoprotein/phosphopeptide interactions with WT PPM1A and PPM1A variants. A, multiple sequence alignment of selected PPM1A substrates. Target phosphoserine/phosphothreonine residues are indicated by lower case, red letters. Classification of flanking residues as hydrophobic (green), polar (lavender), positively-charged (blue), or negatively-charged (pink) is indicated by colored boxes. Lower case letters indicate proximal sites of phosphorylation. B, fluorescence anisotropy titration of fluorescein-labeled p38(175-185, 180pT) with PPM1A D146A at 25 °C. Data represent the mean ± SEM of 4 titrations and were fit to a single site binding model with $K_d = 48 \pm 16 \mu M$. C, NMR chemical shift perturbation study of the binding of p38(175-185, 180pT) to PPM1A D146A at 30 °C. TOSCY spectra of p38(175-185, 180pT) peptide alone (blue contours) or in the presence of PPM1A D146A (red contours, 10:1 molar ratio). D, the primary structure of the cyclic thioether isopeptide, c(MpSlpYVA). E, isothermal titration calorimetry analysis of the binding of c(MpSlpYVA) to PPM1A $\text{cat} D146E$ at 25 °C. Left panel, representative thermal trace for injection of c(MpSlpYVA) into PPM1A $\text{cat} D146E$ solution is shown. Right panel, integrated heats of injection for the binding of c(MpSlpYVA) to PPM1A $\text{cat} D146E$ were fit to a single site binding model after subtraction of peptide heats of dilution. Data represent the mean ± SEM of two titrations.
Figure 2 Structure of the PPM1A_{cat} D146E–c(MpSIpYVA) complex. A, representation of the PPM1A_{cat} (chain A, ribbon representation, sky blue) with three Ca^{2+} ions (green spheres) and trapped cyclic phosphopeptide (chain D, stick representation, sandy brown). The Flap structure (aa 165-219, grey) is adjacent to one end of the active site. Selected active site residue side chains are shown in stick representation. Metal-coordinating water molecules are represented as red balls. The mutated residue, D146E, is located at the apex of the β6-β7 loop. B, Close-in view of the active site. Representations and colorings are as in Panel A. Hydrogen bonds are indicated in magenta. The bridging water molecule/hydroxide ion (asterisk) is jointly coordinated by M1 and M2. M1 is also coordinated by D60, D239, D282, and two water molecules. M2 is also coordinated by D60, G61, one of the phosphate oxygens of the cyclic peptide phosphoserine residue, and two water molecules. M3 is coordinated by D239, D243, and four water molecules. C, Simulated, annealed composite omit electron density map contoured at 1σ (grey mesh) overlayed on stick representation with coloring as in panel A. Localized electron density for the Cδ and Oε atoms of the D146E mutated residue is absent. D, surface representation of electrostatic potential (Coulombic) of PPM1A_{cat} (semi-transparent surface, 10 kcal/mol·e, blue; -10 kcal/mol·e, red) in complex with the cyclic peptide (stick representation, sandy brown). E, surface representation of residue mean hydrophobicity according to the Kyte-Doolittle hydrophobicity scale (4.5, hydrophobic, orange; -4.5, polar, blue).
Figure 3. Conformational differences between the catalytic domain (aa 2-297) of PPM1A and the PPM1A_{cat} D146E‒c(MpSlpYVA) complex. A, ribbon diagram showing overlay of the PPM1A catalytic domain (PDB: 1A6Q, aa 2-297, pink) with bound phosphate ion and PPM1A_{cat} D146E (chain A, sky blue) with bound c(MpSlpYVA) in stick representation (chain D, sandy brown), with selected annotations. Ca^{2+} ions (green) of the complex are rendered as semitransparent to allow visualization of the Mn^{2+} ions (purple) of the phosphate-bound protein structure. B, ribbon diagram showing overlay of the active sites of the free protein and the protein-cyclic peptide complex, with selected annotations. The bridging water molecule/hydroxide ion is marked with an asterisk. Rendering is as in panel A. C, comparison of binding-induced differences in conformation and sequence variation among selected metazoan PPM1A homologues. Plot of the distance between Cα carbons of aligned structures for the phosphate-bound protein (PDB: 1A6Q, aa 2-297) and the protein-cyclic peptide complex (chains A, D) (solid black trace, left axis) and smoothed sequence variation among 150 metazoan PPM1A homologues (red dotted trace, right axis). The locations of insertions and deletions (indels) are indicated by black bars. The positions of the Flap subdomain (grey) and major units of secondary structure (α helix, medium blue; β strand, gold) are indicated. Highly conserved residues in the active site are indicated by colored squares: Arg, blue; Asp, red; Gly, grey.
Figure 4. Solution conformations of free PPM1Acat D146E protein and the PPM1Acat D146E‒c(MpSIpYVA) complex by small angle x-ray scattering and constrained molecular dynamics (MD) simulations. A, observed (cyan and pink dots) and predicted scattering profiles (blue and red curves) of PPM1Acat D146E and PPM1Acat D146E‒c(MpSIpYVA), respectively, at 25 °C. Data represent the means of 30 independent scattering measurements. Predicted curves are based on constrained molecular dynamics simulations. B, plot of rms distance to ensemble mean versus residue number for PPM1Acat D146E (blue curve) and PPM1Acat D146E‒c(MpSIpYVA) complex (red curve) based on 150 structures spanning 290 ns of constrained MD simulations. C, close-in view of the active site representing the overlay of 12 structures spanning 290 ns of constrained MD simulations each for free PPM1Acat D146E (upper left, blue hues), the PPM1Acat D146E‒c(MpSIpYVA) complex (upper right, orange hues), free PPM1Acat D146E (lower left, blue/green hues), the PPM1Acat D146E‒c(MpSIpYVA) complex (lower right, red hues). Side chains for H62 and R186 are shown in stick representation. The cyclic peptide is shown in wire representation. D, distributions of R186 Cα‒M1 distances (upper graph) and R186 Cα‒M2 distances (lower graph) over 290 ns of constrained MD simulations for WT PPM1Acat and PPM1Acat D146E as free proteins and as complexed with c(MpSIpYVA). Simulations include three Mg2+ ions in the active site. For the WT protein, M3 is bound in the D146/D239 subsite, whereas for the D146E protein, M3 is bound in the D239/D243 subsite.
Figure 5. Interactions of substrate and third metal binding in the PPM1A active site. A, the PPM1Acat D146E-c(MpSpY-VA) complex is rendered as semi-transparent (sky blue) and mesh (sandy brown) surfaces overlaid onto ribbon and stick representations of the protein and cyclic peptide, respectively. The cyclic peptide and selected protein side chains display heteroatom coloring. The third metal ion (Ca$^{2+}$, green) is bound between the D239 and D243 side chains and is accessible to solvent. B, schematic diagram representing the random association mechanism for binding of substrate (S) and essential metal ion (M) to form the catalytically competent EMS complex. In the rapid equilibrium approximation, the parameter $\alpha$ reflects the strength of coupling between substrate and metal ion binding. C, dependence of initial rates on p38(175-185, 180pT) concentrations at various Mg$^{2+}$ concentrations for PPM1Acat (left panel) and PPM1Acat D243A (right panel) with curves fitted to the apparent Michaelis-Menten equation by nonlinear least squares. Data represent the means ± S.E.M. of two determinations. D, secondary plots showing the dependence on Mg$^{2+}$ concentration of $V_{\text{app}}$ (left panel) and $K_{m\text{app}}$ (right panel) for PPM1Acat (blue) and PPM1Acat D243A (orange) with curves fitted to the random order, bi-substrate model. Data represent the best-fit values ± S.E.M. of apparent Michaelis constants from non-linear least squares fitting of initial rate data.
A trapped human PPM1A–phosphopeptide complex reveals structural features critical for regulation of PPM protein phosphatase activity
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