Serine/Threonine Phosphatases: Mechanism through Structure

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The reversible phosphorylation of proteins is accomplished by opposing activities of kinases and phosphatases. Relatively few protein serine/threonine phosphatases (PSPs) control the specific dephosphorylation of thousands of phosphoprotein substrates. Many PSPs, exemplified by protein phosphatase 1 (PP1) and PP2A, achieve substrate specificity and regulation through combinatorial interactions between conserved catalytic subunits and a large number of regulatory subunits. Other PSPs, represented by PP2C and FCP/SCP, contain both catalytic and regulatory domains within the same polypeptide chain. Here, we discuss biochemical and structural investigations that advance the mechanistic understanding of the three major classes of PSPs, with a focus on PP2A.

The concept of protein phosphorylation, discovered by Edmond Fischer and Edwin Krebs, arose from the demonstration of a dual requirement for ATP and a "converting enzyme" (subsequently named phosphorylase kinase) in the in vitro conversion of phosphorylase *b* to phosphorylase *a* (Fischer and Krebs, 1955; Krebs and Fischer, 1956; Krebs et al., 1958). This process involved the transfer of a phosphate group from ATP to phosphorylase *b*, and the resulting phosphorylase *a* was found to be a phosphoprotein (Krebs and Fischer, 1956; Sutherland and Wosilait, 1955). Intriguingly, the enzyme that converts phosphorylase *a* back to *b*, called the "PR enzyme" (phosphorylase phosphatase), had been reported a decade earlier (Cori and Green, 1943), although the chemical nature of the reaction remained enigmatic until inorganic phosphate was found to be a product of the reaction (Krebs and Fischer, 1956; Sutherland and Wosilait, 1955).

Decades of research since those initial discoveries have shown that reversible phosphorylation of proteins, executed by kinases and phosphatases, constitutes a major form of signaling and an essential mechanism of regulation in all living organisms. In eukaryotic cells, phosphorylation mainly occurs on three hydroxyl-containing amino acids, serine, threonine, and tyrosine, of which serine is the predominant target. Proteomic analysis of 6600 phosphorylation sites on 2244 human proteins revealed that phosphoserine (pSer), phosphothreonine (pThr), and phosphotyrosine (pTyr) account for 86.4%, 11.8%, and 1.8%, respectively, of the phosphorylated amino acids (Olsen et al., 2006). These numbers are in good agreement with a previous study in which radioactive isotope (^{32}P)-labeled phosphoproteins from chicken cells were isolated, acid hydrolyzed, and analyzed by two-dimensional electrophoresis (Hunter and Sefton, 1980). The fully sequenced human genome is thought to contain 518 putative protein kinases (Johnson and Hunter, 2005; Lander et al., 2001; Venter et al., 2001) that can be classified into two families: 90 tyrosine (Tyr) kinases (PTKs) and 428 serine/threonine (Ser/Thr) kinases (PSKs). The exquisite specificity of signaling and the reversible nature of phosphorylation seem to suggest that there would be similar numbers of protein phosphatases in the human genome. Surprisingly, however, there are only 107 putative protein Tyr phosphatases (PTPs) (Alonso et al., 2004) and far fewer protein Ser/Thr phosphatases (PSPs) (~30). Whereas the numbers of PTKs and PTPs roughly match each other, the number of catalytic subunits of PSPs is an order of magnitude lower than that of PSKs. As will be clear from the discussion below, this dichotomy can be explained by the combinatorial formation of PSP holoenzymes from a shared catalytic subunit and a large number of regulatory subunits.

PSPs comprise three major families: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and the aspartate-based phosphatases represented by FCP/SCP (TFIIF-associating component of RNA polymerase II CTD phosphatase/small CTD phosphatase) (Figure 1). For several members of the PPP family, the catalytic subunit associates with a great variety of regulatory subunits. Representative members of the PPP family include protein phosphatase 1 (PP1), PP2A, PP2B (commonly known as calcineurin), PP4, PP5, PP6, and PP7 (Figure 1). The PPM family includes protein phosphatases dependent on manganese/magnesium ions (Mn^{2+}/Mg^{2+}), such as PP2C and pyruvate dehydrogenase phosphatase. In contrast to PPP, members of the PPM family do not have regulatory subunits but contain instead additional domains and conserved sequence motifs that may help determine substrate specificity. For both PPP and PPM, metal ions play a catalytic and central role through the activation of a water molecule for the dephosphorylation reaction. In contrast, FCP/SCP uses an aspartate-based catalysis mechanism. The only known substrate for FCP/SCP is the C-terminal domain

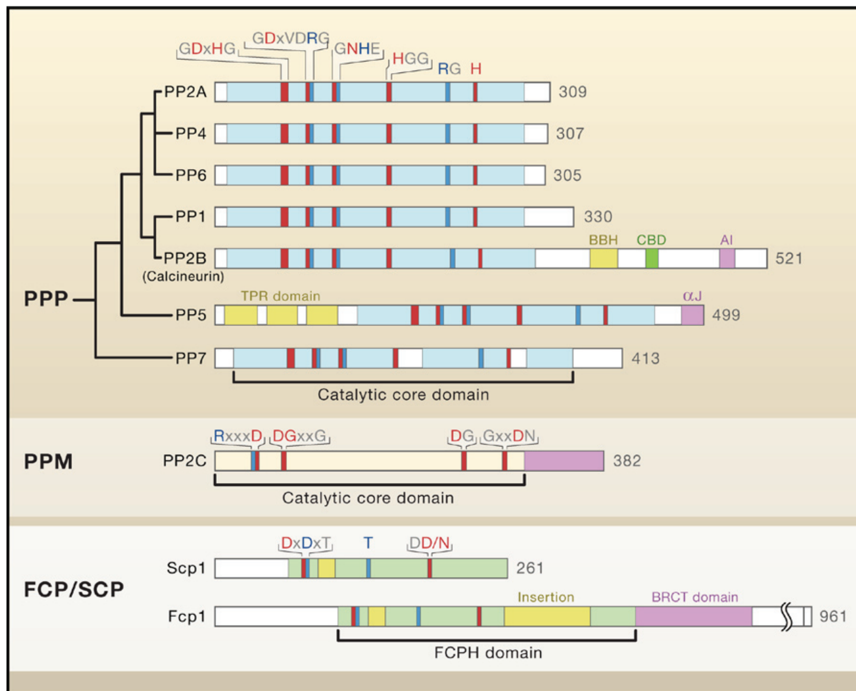


Figure 1. The Three Families of Protein Serine/Threonine Phosphatases

Protein serine/threonine phosphatases (PSPs) can be categorized into three families—phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based phosphatases such as FCP (TFIIF-associating component of RNA polymerase II CTD phosphatase) and SCP (small CTD phosphatase). Representative members of each family are presented here. The catalytic core domains of each protein are indicated below the diagram. Signature sequence motifs are labeled above the diagram. Residues that contribute to metal coordination and phosphate binding are colored in red and blue, respectively. The PPP family contains three characteristic sequence motifs within the conserved 30 kD catalytic domain: GDxHG, GDxVDRG, and GNHE (G, glycine; D, aspartic acid; x, any amino acid; H, histidine; V, valine; R, arginine; N, asparagine; E, glutamic acid). BBH, CNB-binding helix; CTD, carboxy-terminal domain; CBD, Ca^{2+}-calmodulin-binding motif; AI, autoinhibitory sequence; TPR, tetratricopeptide repeat; FCPH, FCP-homology domain. The accession codes for the proteins depicted are as follows: PP1α, P62136; PP2Aα, P67775; PP2Bα, Q08209; PP4, P60510; PP5, P53041; PP6, O00743; PP7, Q9FN02; PP2C, S87759; Fcp1α, NP_004706; Scp1, NP_067021. All proteins here are from *Homo sapiens* except for PP7 (from the model plant *Arabidopsis thaliana*).

(CTD) of RNA polymerase II, which contains tandem repeats of a serine-rich heptapeptide. The conserved structural core of FCP/SCP is the FCP homology (FCPH) domain (Figure 1). FCPs, but not SCPs, contain a BRCT (BRCA1 C-terminal domain like) domain that is C-terminal to the FCPH domain.

Over the last two decades of investigation, cellular functions of PSPs have been documented with increasing detail. An earlier emphasis on functional characterization has recently been complemented by biochemical and structural investigations of all three major families of PSPs, giving rise to major advances in mechanistic understanding. The cellular and physiological functions of PSPs have been extensively discussed in recent reviews (Ceulemans and Bollen, 2004; Cohen, 2002, 2004; Cohen et al., 2005; Gallego and Virshup, 2005; Hinds and Sanchez, 2008; Janssens et al., 2008; Kamenski et al., 2004; Lu and Wang, 2008; Moorhead et al., 2007). Therefore, this review will focus on the molecular mechanisms elucidated by recent biochemical and structural studies.

Protein Phosphatase 1

PP1 is a major protein Ser/Thr phosphatase and is ubiquitously expressed in all eukaryotic cells. PP1 figures prominently in a wide range of cellular processes, including meiosis and cell division, apoptosis, protein synthesis, metabolism, cytoskeletal reorganization, and the regulation of membrane receptors and channels (Ceulemans and Bollen, 2004; Cohen, 2002). Commensurate with its myriad functions, the many forms of PP1 collectively exhibit broad substrate specificity. However, each assembled and functional PP1 enzyme is thought to display stringent substrate specificity and elicits specific biological responses.

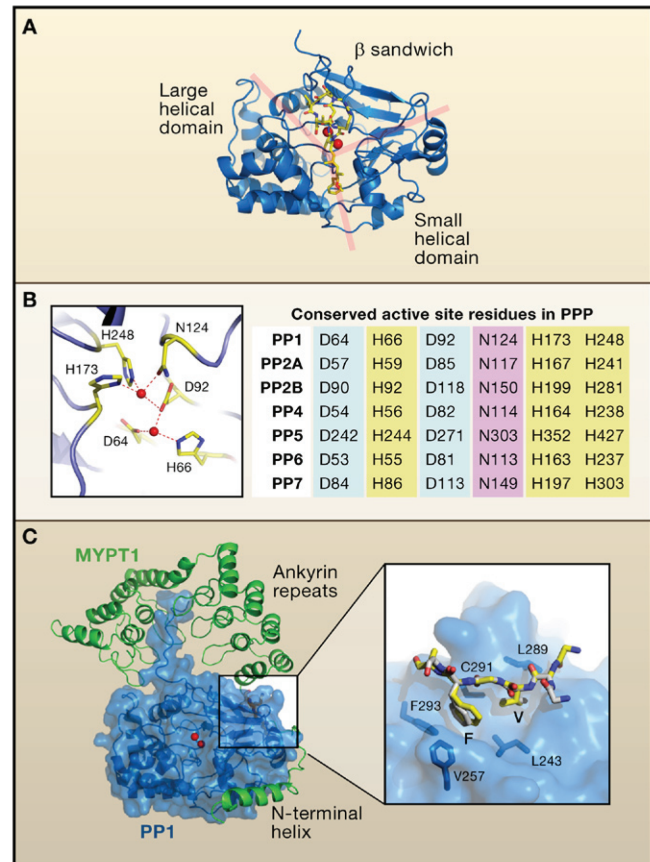
Each functional PP1 enzyme consists of a catalytic subunit and a regulatory subunit (R subunit). The catalytic subunit of PP1 is highly conserved among all eukaryotes, with approximately 70% or greater protein sequence identity in any pairwise alignment. These sequences support a conserved fold and a similarly positioned active site for all members of the PPP family. At least 100 putative PP1-binding R subunits have been identified, with many more expected to be found (Cohen, 2004; Moorhead et al., 2007). Analysis of known R subunits in diverse eukaryotic lineages suggests an explosive growth in the number of R subunits concurrent with the evolution of multicellular organisms (Ceulemans et al., 2002). These R subunits may target the PP1 catalytic subunit to specific subcellular compartment, modulate substrate specificity, or serve as substrates themselves. Thus, the interactions between the catalytic subunit and specific R subunits are central to the functions of PP1.

The catalytic subunit of PP1 adopts a compact α/β fold, with a β sandwich wedged between two α-helical domains (Egloff et al., 1995; Goldberg et al., 1995) (Figure 1A). Two metal ions, identified as Mn^{2+} and Fe^{2+} (iron), are located in the active site at the three-way joint of the β sandwich and the two helical domains. Coordination of these two metal ions is provided by three histidines, two aspartic acids, and one asparagine. These residues are highly conserved in all members of the PPP family (Figure 2B), suggesting a common mechanism of metal-catalyzed reaction in the protein family. The two metal ions are thought to bind and activate a water molecule, which initiates a nucleophilic attack on the phosphorous atom (Egloff et al., 1995; Goldberg et al., 1995). Three shallow surface grooves roughly follow the domain boundaries and converge at the catalytic center, forming a Y-shaped surface feature (Figure 2A). Tumor-inducing toxins such as microcystin and okadaic acid associate tightly with the active site through interactions with amino acids on the surface loops of the catalytic center.

Early studies suggested that most R subunits contain the sequence motif RVxF/W (R, arginine; V, valine; x, any amino acid; F, phenylalanine; W, tryptophan). This notion was supported by the crystal structure of PP1 bound to a peptide containing the sequence RRVSFA (S, serine; A, alanine) (Egloff et al., 1997). The valine and phenylalanine residues of the peptide stack against hydrophobic amino acids on the surface of PP1, whereas the side chain of the first, but not the second, arginine residue makes hydrogen bonds to PP1. Subsequent studies focused on the identification and improvement of the consensus peptide sequence (Croci et al., 2003; Enz and Croci, 2003; Wakula et al., 2003). Most of the R subunits of PP1 were thought to contain a surface motif of [RK][X]$_{0\text{-}1}$[VI]X[F/W] (I, isoleucine) (Wakula et al., 2003). More recently, systematic analysis of the docking peptides that combined biochemistry with molecular dynamics gave rise to a refined consensus sequence of [H/K/R][A/C/H/K/M/N/Q/R/S/T/V][V][C/H/K/N/Q/R/S/T][F/W] (H, histidine; K, lysine; C, cystein; M, methionine; N, asparagine; Q, glutamine; T, threonine) (Meiselbach et al., 2006). This consensus sequence allowed the accurate prediction and experimental confirmation of several previously unknown PP1-binding proteins and reconciled a body of observations. Within this consensus sequence, the most conserved valine and phenylalanine/tryptophan residues appear to anchor the binding of the R subunit to PP1, whereas the other residues provide the needed specificity for recognizing different R subunits.

How do R subunits help improve substrate specificity? Structural analysis of PP1 bound to the myosin phosphatase targeting subunit (MYPT1) protein revealed a tantalizing clue (Terrak et al., 2004). MYPT1 associates with PP1 using a tripartite binding mode. Within MYPT1, a TKVKF sequence interacts with the conserved hydrophobic surface of PP1, an ankyrin repeat domain caps the C terminus of PP1, and a hydrophobic N-terminal helix docks onto the surface of PP1 (Figure 2C). Although the clasping of PP1 by MYPT1 results in no apparent conformational changes, MYPT1 binding does modify the Y-shaped surface groove surrounding the catalytic center of PP1. This was thought to enhance substrate specificity by introducing a new recognition surface for the substrate protein (Terrak et al., 2004). Consistent with this notion, the ankyrin repeats of MYPT1 are important for enhanced substrate specificity for myosin despite relatively weak binding between myosin and isolated ankyrin repeats (Hirano et al., 1997). A definitive proof of this notion requires additional biochemical and biophysical investigation that involves the substrate protein. Comprehensive mechanistic understanding of PP1 will likely require the structural elucidation of PP1 bound to different classes of R subunits whose functions have been biochemically characterized.

The phosphatase activity of PP1 is regulated by a number of endogenous inhibitory proteins such as inhibitor-1 (I-1) (Nimmo and Cohen, 1978), inhibitor-2 (I-2) (Foulkes and Cohen, 1980), CPI-17 (Eto et al., 1997), and DARPP-32 (Walaas and Greengard, 1991). Specific inhibition of PP1 by I-1 and DARPP-32 requires phosphorylation of a conserved threonine residue at the N termini of the inhibitory proteins, which is thought to bind the active site of PP1 (Desdouits et al., 1995). Despite sequence conservation, PP2A and PP2B are not sensitive to inhibition by I-1 or I-2, and this characteristic was the basis for classification



Conserved active site residues in PPP

PP1	D64	H66	D92	N124	H173	H248
PP2A	D57	H59	D85	N117	H167	H241
PP2B	D90	H92	D118	N150	H199	H281
PP4	D54	H56	D82	N114	H164	H238
PP5	D242	H244	D271	N303	H352	H427
PP6	D53	H55	D81	N113	H163	H237
PP7	D84	H86	D113	N149	H197	H303

Figure 2. Structure and Mechanism of Protein Phosphatase 1
(A) Structure of the catalytic subunit (blue) of protein phosphatase 1 (PP1) bound to okadaic acid (OA, yellow ball and stick). A Y-shaped surface groove (pink) is defined by the three domains of PP1. The two metal ions (red spheres) are Mn^{2+} (manganese) and Fe^{2+} (iron).
(B) Conserved coordination of the binuclear metal center in the PPP family phosphatases. The metal-binding scheme of PP1 where six highly conserved amino acids bind to the two metal ions is indicated on the left. The corresponding metal-binding residues from other PPP family members are shown on the right.
(C) Structure of the PP1 catalytic domain (blue) bound to the regulatory subunit myosin phosphatase targeting subunit (MYPT1) protein (green). Three elements of MYPT1 are involved in the interactions: a VxF (V, valin; x, any amino acid; F, phenylalanine) motif that binds to the conserved hydrophobic surface of PP1, an ankyrin repeat domain that caps the C terminus of PP1, and a hydrophobic N-terminal helix that docks onto the surface of PP1. (Inset) A close-up view of the recognition of the VxF motif by PP1.
All structural figures were prepared with PyMol (DeLano, 2002).

of type 1 (PP1) versus type 2 phosphatases (Ingebritsen and Cohen, 1983). By analogy with the PP1 inhibitors, the endogenous inhibitors of PP2A were named I_1^{PP2A} and I_2^{PP2A} (Li et al., 1995) and were found to be putative HLA class II-associated protein I (PHAP-I) (Li et al., 1996a) and SET (Li et al., 1996b), respectively. The mechanism by which PP2A is inhibited by PHAP-I and SET remains to be elucidated.

Calcineurin/Protein Phosphatase 2B

Calcineurin (also known as protein phosphatase 2B or PP2B) plays an important role in numerous calcium-dependent biological processes, including neurodevelopment and memory, immune response, cardiac hypertrophy, signal transduction,

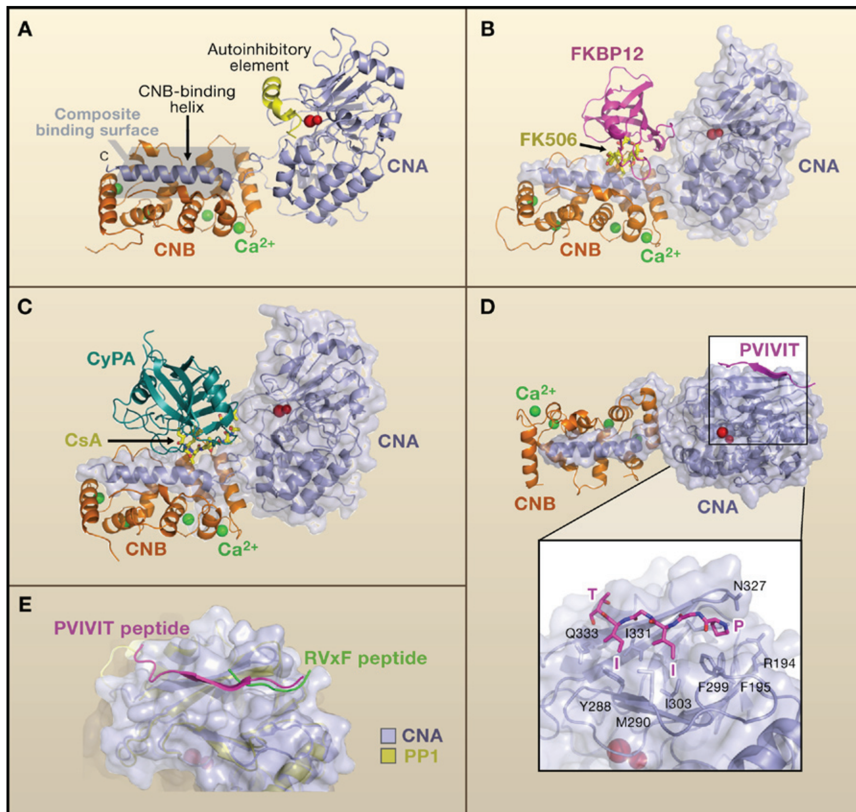


Figure 3. Structure and Mechanism of Calcineurin

(A) Structure of free calcineurin (also called PP2B), which consists of calcineurin A (CNA, blue) and calcineurin B (CNB, gold). The CNB-binding helix (BBH) of CNA and the two calmodulin (CaM)-like domains of CNB together form a composite surface. The autoinhibitory loop forms an α helix and binds the active site of the phosphatase, blocking substrate access. The two catalytic metal ions and the four calcium atoms are colored red and green, respectively.

(B) The FKBP12-FK506 complex binds to the composite surface formed by CNB and the BBH. This binding is thought to inhibit calcineurin-mediated dephosphorylation of nuclear factor of activated T cell (NFAT), a transcription factor, ultimately resulting in the suppression of T cell activation. FK506 (yellow) directly interacts with residues from both CNB and the BBH domain, explaining why interaction between calcineurin and FKBP12 requires FK506. FKBP12 (magenta) interacts with both the phosphatase domain and the BBH-CNB composite surface.

(C) Structure of calcineurin bound to the cyclophilin-cyclosporin A complex.

(D) The substrate peptide PxIxIT (P, proline; x, any amino acid; I, isoleucine; T, threonine) binds to a surface groove on CNA through β-augmentation, where the peptide substrate forms a β strand at the edge of a β sheet in calcineurin. (Inset) The side chains of the proline and isoleucine residues in the PxIxIT motif interact with hydrophobic amino acids on the surface of CNA.

(E) A conserved substrate-binding site on PP1 and calcineurin. The surface groove of CNA occupied by the PxIxIT peptide corresponds to that of PP1 bound by the RVxF peptide.

and muscle development (Rusnak and Mertz, 2000). Calcineurin consists of a catalytic subunit (calcineurin A or CNA) and a regulatory subunit (calcineurin B or CNB). CNA contains an N-terminal phosphatase domain, followed by a CNB-binding helical domain, a calcium (Ca^{2+})-calmodulin-binding motif, and an autoinhibitory element (Figure 1). Calcineurin is inactive alone and only gains phosphatase activity upon association with Ca^{2+}-calmodulin (Ca^{2+}-CaM).

The mechanism of calcineurin autoinhibition was revealed by its crystal structure (Kissinger et al., 1995), which showed that the autoinhibitory element forms an α helix and blocks access to the catalytic center (Figure 3A). The autoinhibitory element interacts with surface amino acids of the phosphatase domain through a combination of hydrogen bonds and van der Waals contacts. This structural feature suggests that displacement of the autoinhibitory element may be required for the activation of the protein. The phosphatase domain of CNA is structurally similar to the catalytic subunit of PP1, with the same pattern of metal ion coordination (Griffith et al., 1995; Kissinger et al., 1995). The two metal ions associated with the protein were identified as Zn^{2+} (zinc) and Fe^{3+}. CNB consists of a pair of Ca^{2+}-binding domains, each containing two EF-hand motifs. All four calcium-binding sites in CNB are fully loaded, with each calcium ion coordinated by five oxygen atoms—four from side chains and one from a main chain carbonyl group. The two Ca^{2+}-binding domains of CNB are organized around the CNB-binding helical domain (BBH).

The hydrophobic face of the BBH helix is buried in a greasy pocket on the surface of CNB, whereas the other exposed face of BBH forms a composite binding surface with neighboring residues of CNB (Figure 3A). Both the FKBP12-FK506 (Griffith et al., 1995; Kissinger et al., 1995) and the cyclophilin A (CyPA)-cyclosporin A (CsA) (Huai et al., 2002; Jin and Harrison, 2002) complexes associate with this surface through a similar set of interactions (Figures 3B and 3C), despite a lack of structural homology between these two complexes. Binding by these immunosuppressant complexes is thought to inhibit calcineurin-mediated dephosphorylation of the transcription factor nuclear factor of activated T cell (NFAT), ultimately resulting in the suppression of T cell activation. In both cases, the immunosuppressants make direct interactions with residues from both CNB and the BBH domain. This observation explains why interactions between calcineurin and immunophilins strictly depend on the presence of the immunosuppressants. The immunophilins directly interact with both the phosphatase domain and the BBH-CNB composite surface, with approximately 80% of the interface residues of calcineurin involved in binding to both FKBP12-FK506 and CyPA-CsA (Ke and Huai, 2003). Differences in hydrogen bond interactions appear to dictate the specific recognition of distinct immunosuppressant complexes, and these differences provide plausible explanation for the observed immunosuppressant-specific phenotypes. For example, the mutation of tyrosine 341 to phenylalanine (Y341F) in calcineurin rendered T lymphocytes and yeast cells resistant to CsA, but not to FK506 (Cardenas et al., 1995; Zhu et al., 1996). In the structure, CsA, but not FK506, makes two hydrogen bonds to the hydroxyl group of tyrosine 341 in calcineurin; the Y341F mutation eliminates these interactions, likely resulting in the weakened interaction between the CyPA-CsA complex and calcineurin.

How does the immunophilin-immunosuppressant complex inhibit the phosphatase activity of calcineurin? An intuitive explanation is that the relatively close proximity of the bound immunophilin-immunosuppressant complex to the catalytic center of calcineurin may hinder substrate access (Griffith et al., 1995). It is also possible that the composite surface above BBH, which is occupied by the immunosuppressant complex, is involved in substrate recognition. In addition, activation of calcineurin requires Ca^{2+}-CaM; yet the Ca^{2+}-CaM-binding element in the protein is positioned C-terminal to BBH (Figure 1). Binding of the immunosuppressant complex to the composite surface involving the BBH domain may negatively affect the ability of Ca^{2+}-CaM to activate the phosphatase domain of calcineurin.

How does calcineurin recognize substrate proteins? Extensive studies of calcineurin substrates in yeast and mammalian cells, especially on NFAT1, revealed a consensus recognition motif of PxIxIT (P, proline) (Bultynck et al., 2006; Czirjak and Enyedi, 2006; Czirjak et al., 2004; Heath et al., 2004; Li et al., 2004; Roy et al., 2007). Sequence variations within the PxIxIT motif results in a wide range of binding affinities—between 0.5 and 250 μM—to calcineurin, (Li et al., 2007). Structural analysis of calcineurin bound to the peptide PVIVIT (V, valine) helped to explain the findings of these biochemical studies (Li et al., 2007; Takeuchi et al., 2007). The PVIVIT peptide forms a β strand with strand β14 of CNA through main chain hydrogen bonds (Figure 3D). The side chains of the proline and the two isoleucine residues in the PVIVIT motif interact with hydrophobic amino acids on the surface of CNA (Figure 3D), whereas the side chain of threonine makes a hydrogen bond to aspartic acid 330 of CNA. In addition to the overall structural similarity between PP1 and calcineurin, the surface groove of CNA that accommodates the PVIVIT peptide corresponds to the surface groove in PP1 that receives the RVxF peptide (Figure 3E). Although the presence of the PxIxIT motif is necessary for substrate recognition, additional binding elements from the substrate may be required for the specific activity of calcineurin.

How does Ca^{2+}-CaM activate calcineurin? Despite rigorous efforts, a clear answer to this question remains at large. A structure of a complex between calcineurin and Ca^{2+}-CaM may prove essential for deciphering this puzzle. Nonetheless, biochemical and structural investigation has revealed an intriguing clue. Although Ca^{2+}-CaM exists as a monomer, the Ca^{2+}-CaM-binding motif of calcineurin was shown to induce the formation of a stable dimer of Ca^{2+}-CaM (Ye et al., 2008). Structural analysis reveals that the Ca^{2+}-CaM-binding motif of calcineurin forms a contiguous α helix, which organizes two Ca^{2+}-CaM molecules into a head-to-tail dimer (Ye et al., 2008). This observation suggests that calcineurin may form a dimer upon activation by Ca^{2+}-CaM. Consistent with this notion, two molecules of calcineurin were found to interact with a single peptide substrate through β-augmentation, where the peptide substrate forms a β strand at the edge of a β sheet in calcineurin (Li et al., 2007). Biochemical analysis and modeling studies further suggested that it is possible for Ca^{2+}-CaM, calcineurin, and the substrate to form a 2:2:1 complex (Ye et al., 2008).

Protein Phosphatase 2A

Protein phosphatase activities that regulate cellular metabolism are attributed to two types: type 1, namely PP1, and type 2, which consisted of three enzymes called PP2A, PP2B, and PP2C (Ingebritsen and Cohen, 1983). In the late 1980s, this nomenclature was accepted and the catalytic subunits of PP2A were cloned (Arino et al., 1988; Green et al., 1987; Stone et al., 1987). PP2A plays an important role in development, cell proliferation and death, cell mobility, cytoskeleton dynamics, the control of the cell cycle, and the regulation of numerous signaling pathways (Janssens and Goris, 2001); it is also likely to be an important tumor suppressor (Janssens et al., 2005; Mumby, 2007). One of the most abundant enzymes, PP2A accounts for up to 1% of total cellular protein in some tissues. PP2A is highly conserved from yeast to humans, and its regulatory mechanism is extraordinarily complex. Cellular PP2A exists in two general forms—a heterodimeric core enzyme and a heterotrimeric holoenzyme. The PP2A core enzyme consists of a scaffold subunit (also known as the A or PR65 subunit) and a catalytic subunit (C subunit). The scaffold and the catalytic subunits each have two isoforms, α and β, with the α isoform being about 10-fold more abundant than the β isoform. The PP2A core enzyme interacts with a variable regulatory subunit to assemble into a holoenzyme. The regulatory subunits comprise four families: B (also known as B55 or PR55), B′ (B56 or PR61), B″ (PR48/PR72/PR130), and B‴ (PR93/PR110). Each family consists of two to five isoforms that are encoded by different genes; some isoforms have multiple splice variants. For example, the B′ family contains five isoforms, α, β, γ, δ, and ε. The human B′γ isoform has at least three different splice variants called γ1, γ2, and γ3. Except for subunits of the B‴ family, all members of these regulatory subunit families have been shown to bind directly to the PP2A core enzyme. Although highly conserved within the same family, these regulatory subunits share little sequence similarity across families, and their expression levels vary greatly in different cell types and tissues.

The PP2A Core Enzyme

The PP2A scaffold subunit contains 15 tandem HEAT (huntingtin-elongation-A subunit-TOR) repeats, which form an elongated, horseshoe-shaped structure (Groves et al., 1999). Each HEAT repeat comprises a pair of antiparallel α helices, with the interhelical loop composed of highly conserved sequences. A contiguous ridge is formed by the 15 interhelical loop sequences. Structural analysis revealed that the catalytic subunit recognizes the conserved ridge of HEAT repeats 11–15 (Xing et al., 2006) (Figure 4A). Two tumor-derived missense mutations, R418W in the α isoform and V545A in the β isoform of the scaffold subunit (Ruediger et al., 2001b; Wang et al., 1998), map to the interface, suggesting a plausible explanation to the observation that these mutations crippled interaction of the scaffold with the catalytic subunit (Ruediger et al., 2001a, 2001b). Although other PPP family members share extensive sequence similarity with the catalytic subunit of PP2A, they do not associate with the PP2A scaffold subunit. Structural analysis of the interface between the catalytic and scaffold subunits provides a satisfying explanation to this observation. Among the amino acids of the PP2A catalytic subunit that specifically

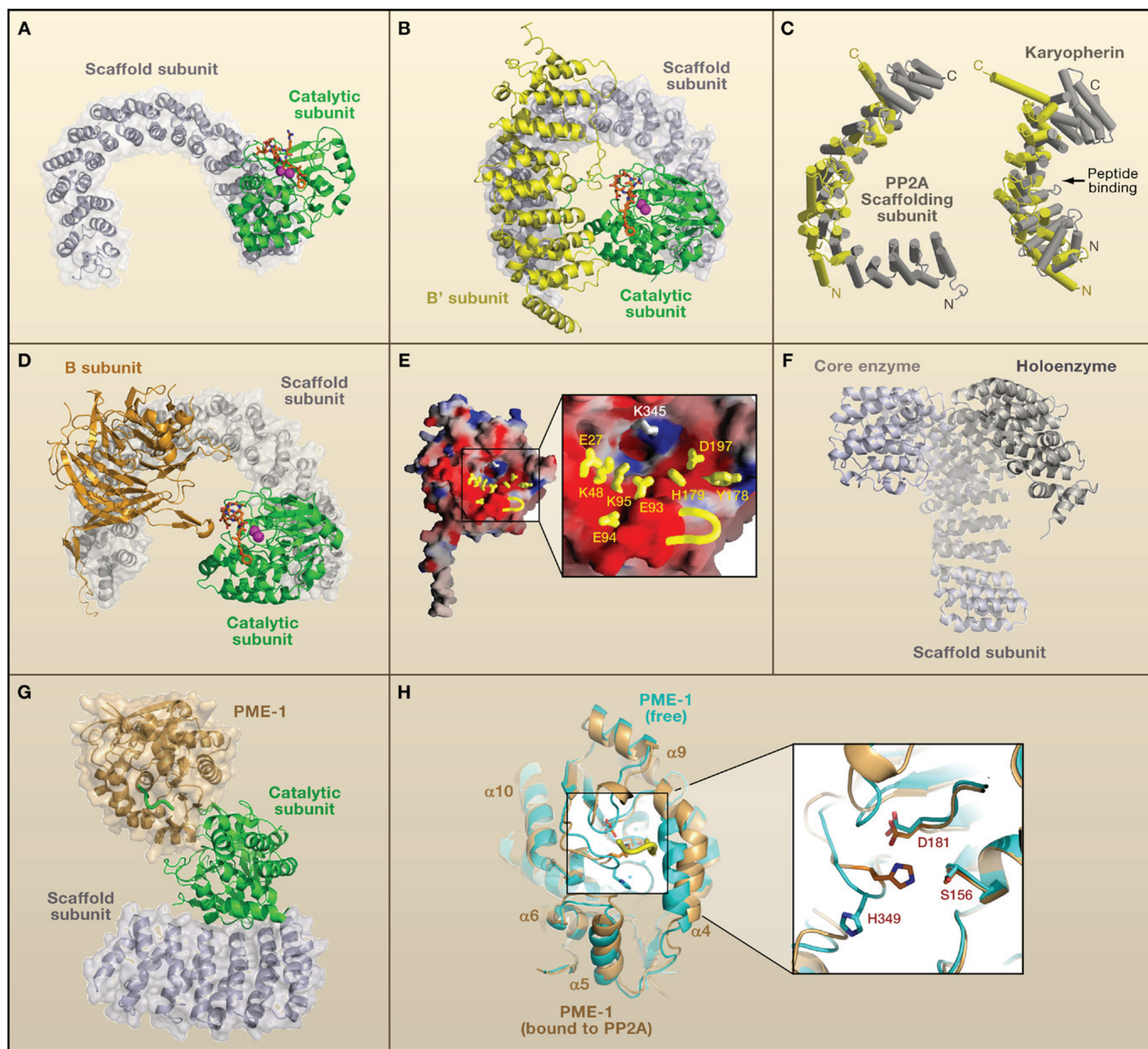


Figure 4. Assembly, Structure, and Mechanisms of PP2A

(A) Structure of the heterodimeric PP2A core enzyme. The catalytic subunit (green) binds to HEAT (huntingtin-elongation-A subunit-TOR) repeats 11–15 of the scaffold subunit (gray).

(B) Structure of the heterotrimeric PP2A holoenzyme involving the B′ regulatory subunit. The B′ subunit (yellow) recognizes HEAT repeats 2–8 of the scaffold subunit and makes extensive interactions with the catalytic subunit.

(C) The B′ subunit is structurally similar to the scaffold subunit of PP2A and the nuclear transport protein karyopherin. B′ contains eight HEAT-like repeats and has a concave surface that may be responsible for substrate recognition.

(D) Structure of a heterotrimeric PP2A holoenzyme harboring the B regulatory subunit. The B subunit (gold) recognizes HEAT repeats 1–7 and makes few interactions with the catalytic subunit.

(E) The B subunit contains an acidic top face for interaction with the microtubule-binding Tau protein. Eight residues (glutamate 27, E27; lysine 48, K48; glutamate 93; glutamate 94; lysine 95; tyrosine 178, Y178; histidine 179, H179; aspartate 197, D197) that are important for binding to Tau are labeled, with their side chains in yellow. In addition, a contiguous stretch of seven amino acids (from phenylalanine 84 to leucine 90) important for binding to Tau are indicated only by the main chain (yellow). Mutation of these seven amino acids also resulted in compromised binding to Tau.

(F) Conformational flexibility of the scaffold subunit of PP2A. Shown here is a comparison between the scaffold subunit (purple), taken from the core enzyme structure, and the scaffold subunit (gray), taken from the B′-containing holoenzyme structure.

(G) Structure of the PP2A methylesterase PME-1 (brown) bound to the heterodimeric PP2A core enzyme. PME-1, which catalyzes the removal of PP2A methylation, interacts only with the catalytic subunit of PP2A (green).

(H) Conformational changes of PME-1 upon binding to the PP2A core enzyme. A structural comparison of free PME-1 (cyan) with that bound to the PP2A core enzyme (brown) shows that helix α4 is shifted in the bound structure, thus allowing the accommodation of the C-terminal peptide of PP2A catalytic subunit.

(I) Formation of an active site in PME-1 upon binding to the PP2A core enzyme. (Inset) Compared to free PME-1, the side chain of histidine 349 (H349) in the PP2A-bound PME-1 is translocated by 8 Å, within hydrogen bond distance of aspartate 181 (D181) and serine 156 (S156).

interact with the scaffold subunit, most have been replaced by nonconserved amino acids in PP1, PP2B, PP5, and PP7. The only exceptions are PP4 and PP6, which contain the majority, but not all, of these interface residues.

The catalytic subunit of PP2A is the primary target of a number of potent tumor-inducing toxins, such as okadaic acid (OA) and microcystin-LR (MCLR). OA has an inhibitory constant of approximately 0.1 nM for the phosphatase activity of PP2A, which is about 100-fold more potent than its inhibitory constant for PP1 (MacKintosh et al., 1990). Both OA and MCLR interact with a similar set of amino acids surrounding the active site of the catalytic subunit (Xing et al., 2006). Structural comparison revealed that a hydrophobic cage in the catalytic subunit of PP2A that accommodates OA is less well formed in PP1 (Xing et al., 2006), which may account for the observed differences in inhibition.

The PP2A Holoenzyme

The heterotrimeric PP2A holoenzyme is believed to exhibit exquisite substrate specificity as well as spatially and temporally determined functions. For example, the B′ subunit, but not B or B″, was thought to be specific for interacting with shugoshin (Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006), a centromeric protein required for proper genome segregation (Gregan et al., 2008). In contrast, the B, but not B′ or B″, subunit was responsible for dephosphorylation of the microtubule-binding protein Tau (Drewes et al., 1993; Gong et al., 1994; Xu et al., 2008). Thus structural elucidation of a PP2A holoenzyme was expected to unveil major insight into how a regulatory subunit facilitates specific PP2A function. The structure of the B′-containing PP2A holoenzyme revealed that the regulatory subunit makes extensive interactions with both the scaffold subunit, through HEAT repeats 2–8, and the catalytic subunit (Cho and Xu, 2006; Xu et al., 2006) (Figure 4B). The B′ subunit shows unanticipated structural mimicry to the scaffold subunit and contains eight HEAT-like repeats (Figure 4C).

The structure of the B′ subunit also resembles that of several other superhelical proteins, including karyopherin, which is involved in transporting proteins through the pores of the nuclear envelope, and β-catenin, a component of the Wnt signaling pathway (Figure 4C). In the assembled PP2A holoenzyme, the convex surface of B′ associates with the scaffold subunit, and the acidic, concave surface is tilted toward the active site pocket of the PP2A catalytic subunit. Similar to karyopherin and β-catenin (Conti et al., 1998; Graham et al., 2000), the B′ subunit may use this surface to recognize a substrate protein for its dephosphorylation.

The structure of PP2A holoenzyme harboring the B subunit shows that the B subunit contains seven WD40 repeats, a β-hairpin handle, and several additional secondary structural elements that are located on the top face of the β-propeller (Xu et al., 2008) (Figure 4D). The bottom face of the β-propeller binds to the ridge of HEAT repeats 3–7, and the β-hairpin handle interacts with HEAT repeats 1 and 2. Unlike the PP2A holoenzyme containing the B′ subunit, the B subunit makes few interactions with the catalytic subunit. A shared feature of the two structurally characterized PP2A holoenzymes is that the potential substrate-binding site is on the top face of the regulatory subunit and close to the active site of the catalytic subunit. This feature supports the notion that a major function of the regulatory subunits is to target substrate phosphoproteins to the phosphatase activity of PP2A. However, these two families of regulatory subunit are unrelated by sequence or by structure, and their specific interactions with the scaffold subunit are quite different.

Substrate Recognition and Targeting

Understanding the function of phosphatases ultimately requires elucidation of the mechanistic underpinnings of substrate dephosphorylation. The available structural information for the PP2A holoenzymes greatly facilitates this undertaking, as exemplified by studies of the microtubule-binding protein Tau (Xu et al., 2008). Hyperphosphorylated Tau is thought to polymerize into neurofibrillary tangles in the brain and contributes to the onset of Alzheimer's disease (Goedert and Spillantini, 2006). A key function of PP2A is to dephosphorylate the hyperphosphorylated Tau protein (Bennecib et al., 2000; Gong et al., 2000; Kins et al., 2001) This activity appears to be mediated by the B family of regulatory subunits (Drewes et al., 1993; Gong et al., 1994). Using an in vitro dephosphorylation assay and structure-guided mutagenesis, the respective binding elements in the B subunit and in Tau were identified (Xu et al., 2008). This study uncovered two nonoverlapping fragments of Tau (both within the microtubule-binding repeats) that interact with the acidic top face of the B subunit (Figure 4E). Because Tau is frequently hyperphosphorylated on a number of amino acids throughout its primary protein sequences, the presence of two PP2A-binding elements likely allows Tau to efficiently target nearby phosphoresidues to the catalytic subunit.

Flexibility of the PP2A Scaffold Subunit

The limited packing interactions between adjacent HEAT repeats and the elongated shape of the scaffold subunit suggest conformational flexibility. This property has been demonstrated by structures of distinct PP2A complexes. Compared to the free scaffold subunit (Groves et al., 1999), HEAT repeats 13–15 are shifted by 20–30 Å upon binding to the catalytic subunit, with the most drastic change between HEAT repeats 12 and 13 (Xing et al., 2006). Compared to the core enzyme, formation of holoenzyme harboring the B′ subunit forces the N-terminal HEAT repeats to twist and move by as much as 50–60 Å (Cho and Xu, 2006; Xu et al., 2006) (Figure 4F). These structural changes, originating from a five-residue sequence in HEAT repeat 11, rearrange the hydrophobic core both between and within the HEAT repeats. The remarkable conformational flexibility of the scaffold subunit likely underlies PP2A function and may be a prerequisite for binding to the catalytic and the regulatory subunits. Certain degrees of flexibility in the scaffold subunit may facilitate the phosphatase activity of the catalytic subunit through improved targeting of the substrate protein.

Reversible Methylation of PP2A

Reversible methylation of the PP2A core enzyme is a conserved regulatory mechanism for PP2A function. Methylation of the C-terminal leucine 309 in a conserved $TPDYFL^{309}$ motif (D, aspartic acid; Y, tyrosine; L, leucine) of the catalytic subunit was shown to enhance the affinity of the PP2A core enzyme for some, but not all, regulatory subunits (Ikehara et al., 2007; Ogris et al., 1997; Tolstykh et al., 2000; Wei et al., 2001; Xing et al., 2006; Xu et al., 2006, 2008). Thus, changes in

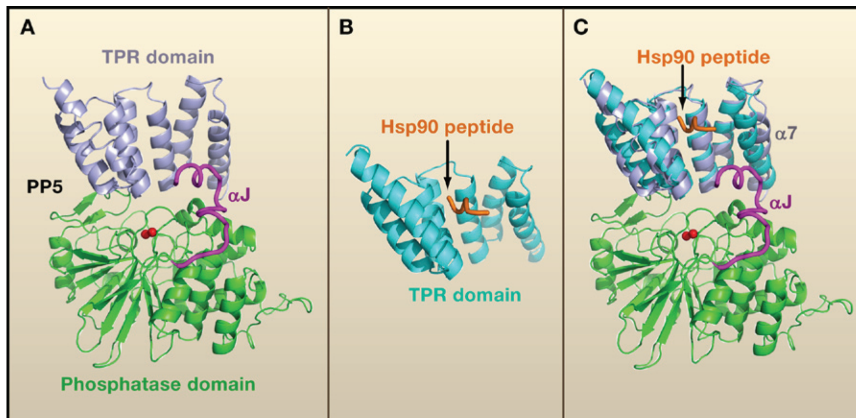


Figure 5. Structure and Mechanism of PP5

(A) The structure of PP5 reveals a mechanism of autoinhibition. The C-terminal αJ helix (magenta) of PP5 binds to its N-terminal TPR domain (purple) and significantly strengthens the interaction between the tetratricopeptide repeat (TPR) domain, a protein-protein interaction motif, and the phosphatase domain (green). These interactions maintain PP5 in an inhibitory state. The catalytic metal ions are colored red.

(B) Structure of the PP5 TPR domain bound to a high-affinity peptide (orange) derived from the protein chaperone Hsp90.

(C) The binding locations for the αJ helix and the Hsp90 peptide do not overlap with each other. However, the two conformations of the TPR domain bound to the αJ helix or the Hsp90 peptide are markedly different from each other. This allostery may explain the mutual exclusion of αJ helix and the Hsp90 peptide binding.

PP2A methylation might modulate the specificity and activity of PP2A in cells. Reversible methylation of PP2A is catalyzed by two conserved and PP2A-specific enzymes, leucine carboxyl methyltransferase (LCMT1) (De Baere et al., 1999; Lee and Stock, 1993) and PP2A methylesterase (PME-1) (Lee et al., 1996). PME-1 catalyzes the removal of the methyl group, thus reversing the activity of LCMT1 (Lee et al., 1996).

Compelling evidence demonstrated that methylation plays an important, perhaps indispensable, role for the assembly of PP2A holoenzymes in cells (Bryant et al., 1999; Gentry et al., 2005; Kloeker et al., 1997; Longin et al., 2007a; Tolstykh et al., 2000; Wei et al., 2001; Wu et al., 2000; Yu et al., 2001). In these studies, formation of the PP2A holoenzyme was examined in cellular extracts. In contrast, several recent investigations relied on recombinant proteins and demonstrated that methylation of the catalytic subunit was dispensable for the in vitro assembly of PP2A holoenzymes harboring the B or B′ subunits (Ikehara et al., 2007; Xu et al., 2006, 2008). Neither mutation of the C-terminal leucine residue nor removal of the 14 C-terminal amino acids in the catalytic subunit prevented formation of heterotrimeric holoenzymes involving the B or B′ subunits (Ikehara et al., 2007; Xu et al., 2006, 2008). Other studies also suggested that methylation was not required for in vitro assembly of the PP2A holoenzyme involving the B′ subunit (Gentry et al., 2005; Longin et al., 2007a).

How can these seemingly conflicting observations be reconciled? One potential explanation is that although methylation may not be required for the assembly of PP2A holoenzymes, it makes a contribution by enhancing binding affinity, which is sufficient to favor holoenzyme assembly in cells. Consistent with this notion, competitive binding experiments between methylated and unmethylated PP2A core enzymes suggested that the methylated core enzyme exhibited a higher binding affinity for the B subunit in comparison to the unmethylated core enzyme (Xu et al., 2008). Another possibility is that methylation may serve as an assembly signal for the PP2A holoenzyme. The methylated C terminus of the catalytic subunit may allow it to be targeted to specific cellular location for holoenzyme assembly. In addition, the methylated C terminus may recruit other proteins that facilitate the assembly of the PP2A holoenzymes within the cell. Examination and characterization of mutant mice that express a catalytic subunit lacking leucine 309 may give insight into the functional importance of the reversible methylation of PP2A in vivo.

How does PME-1 regulate the activity of PP2A? Structural and biochemical analysis revealed two striking consequences for the formation of the heterotrimeric PME-1-PP2A complex (Xing et al., 2008) (Figure 4G). First, binding of the PP2A core enzyme to PME-1, which is catalytically inactive by itself, results in its activation through rearrangement of the catalytic triad, which consists of serine 156, aspartate 181, and histidine 349 (Figures 4H and 4I). The side chain of histidine 349 translocates by 8 Å to be in register with aspartic acid 181 and serine 156 (Figure 4I). The extensive interactions at the PME-1-PP2A interface also lead to the enlargement of the active site pocket of PME-1 to accommodate the C-terminal peptide of the catalytic subunit. Second, the catalytic subunit of PP2A is inactivated by PME-1, not just through demethylation but also through the loss of the catalytic metal ions (Xing et al., 2008). In the structure, the two Mn^{2+} ions in the catalytic center of the PP2A core enzyme were dislodged, likely due to steric hindrance from the conserved residue methionine 335 in PME-1 (Xing et al., 2008). The biological significance of PME-1-mediated PP2A inactivation in cells remains to be investigated (Longin et al., 2004, 2007b).

The interaction between PME-1 and PP2A is subject to regulation by an array of other PP2A-binding factors, including but not limited to LCMT1 and PTPA. In contrast to PME-1, which inactivates PP2A, PTPA and LCMT1 activate PP2A. Formation of a stable complex between PP2A and PME-1 probably blocks LCMT1-catalyzed methylation. Deletion of PTPA homologs (Rrd1/Rrd2) in yeast resulted in elevated levels of stable PP2A-PME-1 complexes and decreased methylation (Hombauer et al., 2007). The dual roles of PME-1 in counteracting the function of PTPA and LCMT1 provide a mechanism for coupling PP2A activation with methylation. The opposing functions of PME-1 and PTPA might form a regulatory circuit for PP2A inactivation and activation, likely through the removal and reloading of the catalytic metal ions.

Protein Phosphatase 5

Whereas most PPP family members have isoforms encoded by different genes, protein phosphatase 5 (PP5) is encoded by a single gene throughout *Eukaryota*. Another unique characteristic of PP5 is that its regulatory and catalytic domains are all contained within the same polypeptide. PP5 is expressed in all

mammalian tissues examined, with high levels in the brain. It regulates cellular proliferation, differentiation, migration, survival and death, and DNA damage repair (Hinds and Sanchez, 2008). In particular, PP5 plays an important role in hormone- and stress-induced signaling. For example, PP5 modulates glucocorticoid receptor (GR) signaling, which controls a range of physiological functions, including development, metabolism, and reproduction, through direct interactions with the Hsp90-GR complex (Davies et al., 2005; Golden et al., 2008). GR proteins not associated with ligand are retained in the cytoplasm, whereas the binding of GR to glucocorticoid results in its release from the Hsp90 complex and subsequent translocation into the nucleus, where it binds to DNA and modulates gene transcription.

PP5 contains a regulatory domain at its N terminus—the tetratricopeptide repeat (TPR) domain, a known protein-protein interaction motif (Figure 1). Similar to calcineurin, the phosphatase activity of free PP5 is suppressed, because the TPR domain and a C-terminal helix αJ together maintain PP5 in an autoinhibited conformation. Interactions with the TPR domain by Hsp90 and fatty acids such as arachidonic acid lead to release of autoinhibition. Structural analysis of human PP5 reveals that access to the active site of the phosphatase domain is blocked by the TPR domain (Yang et al., 2005) (Figure 5A). Association between the TPR domain and the phosphatase domain is strengthened by the αJ helix of PP5, which directly binds to a surface groove on the TPR domain. A peptide derived from Hsp90 interacted with the isolated TPR domain more tightly than with the intact PP5 protein, suggesting that the αJ helix partially blocks Hsp90 binding (Yang et al., 2005). This observation supports a mutual exclusion model, in which steric clash between Hsp90 and the αJ helix, upon binding to the TPR domain, was thought to be responsible for the release of PP5 autoinhibition (Yang et al., 2005). However, structural comparison of the TPR domain bound to a high-affinity Hsp90 peptide (Figure 5B) with PP5 revealed no steric clash between the Hsp90 peptide and the αJ helix (Cliff et al., 2006). Hsp90 binding appears to induce pronounced conformational shift in the TPR domain, especially in the α7 helix (Figure 5C), which may no longer be compatible with binding by the αJ helix (Cliff et al., 2006). This allosteric model is consistent with all published evidence.

The ability of the TPR domain to undergo ligand-induced conformational change allows PP5 to respond to a number of cellular factors and thus may be crucial to PP5 function. For example, PP5 were found to interact, through its TPR domain, with the G proteins $G\alpha_{12}$ and $G\alpha_{13}$ (Yamaguchi et al., 2002) and the small GTPase Rac (Gentile et al., 2006). These interactions were found to stimulate the phosphatase activity of PP5, which subsequently modulates the cognate signaling processes. The phosphatase activity of the full-length PP5, but not the phosphatase domain, is stimulated by polyunsaturated, long-chain fatty acids such as arachidonic acid. Fatty acids, especially those with chain length of 16 carbon atoms or more, directly interact with the TPR domain. These interactions, such as those with arachidonoyl-CoA, were found to alter the conformation of the PP5 TPR domain, which presumably results in the release of autoinhibition (Yang et al., 2005). Because cellular long-chain fatty acyl-CoA esters are known to play a role in the regulation of insulin secretion and gene expression, it is possible that such effects are in part mediated by activated PP5.

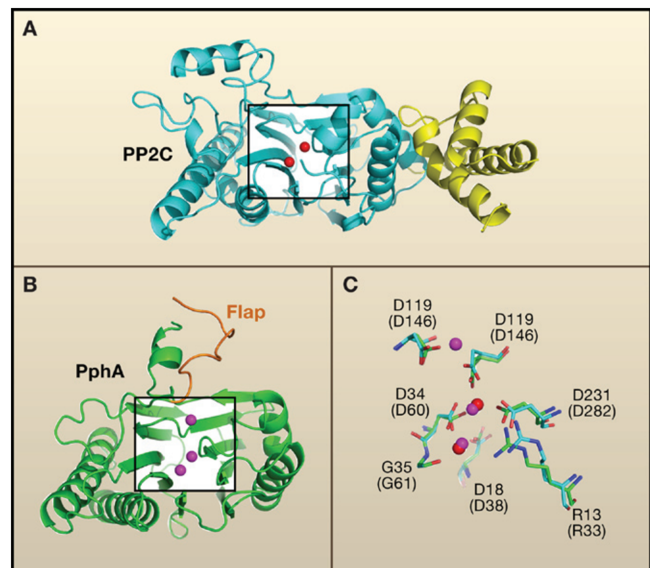


Figure 6. Structure and Mechanism of PP2C
(A) The structure of PP2C, a metal-dependent protein phosphatase (PPM) family member, containing the phosphatase domain (cyan) and the C-terminal helical domain (yellow).
(B) Structure of the prokaryotic PPM homolog PphA from the bacterium *Thermosynechococcus elongates*. In contrast to PP2C, three metal ions (magenta) are located in the active site. A surface loop (flap, orange) close to the active site was shown to play an important role in regulating substrate access to the catalytic center.
(C) The highly conserved metal binding coordination between mammalian PP2C and phosphatase homologs from *prokaryota*. All residues (green) shown are from *Thermosynechococcus elongates*; residues (cyan) indicated in parentheses are from the human protein.

Protein Phosphatase 2C

PP2C and pyruvate dehydrogenase phosphatases belong to the Mn^{2+}/Mg^{2+}-dependent PPM family. In contrast to the PPP family phosphatases, PP2C is insensitive to inhibition by okadaic acid or microcystin. PP2C represents a large family of highly conserved protein phosphatases, with 16 distinct PP2C genes in the human genome that give rise to at least 22 different isoforms (Lammers and Lavi, 2007). Plants contain even more PP2C genes, with 80 and 78 in the model plant *Arabidopsis thaliana* and rice, respectively (Xue et al., 2008). The primary function of PP2C appears to be the regulation of stress signaling, although it also plays a role in cell differentiation, growth, survival, apoptosis, and metabolism (Lu and Wang, 2008). Some PP2C members, such as PP2Cα, PP2Cβ, and PH domain leucine-rich repeat protein phosphatase (PHLPP), are candidate tumor suppressor proteins, whereas others, such as PP2Cδ (also known as Wip1), may contribute to oncogenic transformation.

The conserved catalytic core domain of human PP2C contains a central β sandwich, with each β sheet flanked by a pair of α helices (Das et al., 1996) (Figure 6A). This arrangement

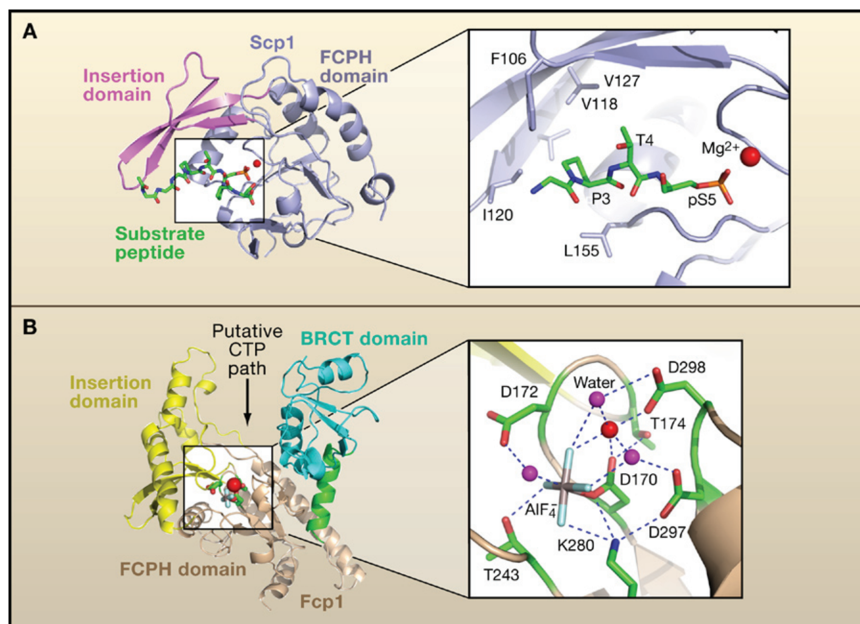


Figure 7. Structure and Mechanism of FCP/SCP

(A) Recognition mechanism of a C-terminal domain (CTD) phosphopeptide from RNA polymerase II by the small CTD phosphatase (Scp1). The specific interactions mainly involve binding of the proline at the third position (Pro3) in the heptad repeat sequence YSPTSPS in the CTD by hydrophobic amino acids from Scp1 and the coordination of the phosphate group on the phosphorylated serine in the fifth position (pSer5) in the repeat by the Mg^{2+} ion.

(B) The structure of Fcp1 (TFIIF-associating component of RNA polymerase II CTD phosphatase), which like Scp1 also dephosphorylates the CTD of RNA polymerase II, reveals a Y-shaped architecture. The BRCT domain (cyan) of Fcp1 does not bind to the phosphopeptide as previously thought. Rather, the CTD peptide is likely accommodated by the deep canyon between the two arms of the Y-shaped molecule.

generates a cleft between the two β sheets, with the two metal ions located at the base of the cleft. Each metal ion is hexacoordinated by amino acids and water molecules. Similar to members of the PPP family, dephosphorylation is thought to involve nucleophilic attack of the phosphorous atom by a metal-activated water nucleophile through an S_N2 mechanism. Three additional α helices, unique to PP2C, associate with the core domain on one side (Figure 6A) and may contribute to substrate specificity or regulation.

Genome analysis of the bacterial kingdom, particularly cyanobacteria, revealed a large number of PPM homologs (Shi, 2004; Zhang et al., 2005; Zhang and Shi, 2004). This observation suggests that reversible protein serine/threonine phosphorylation may play an important role in bacterial physiology. Structural analysis of these PPM homologs has been performed for PphA from *Thermosynechococcus elongates* (Schlicker et al., 2008) (Figure 6B), PstP from *Mycobacterium tuberculosis* (Pullen et al., 2004), MspP from *Mycobacterium smegmatis* (Bellinzoni et al., 2007; Wehenkel et al., 2007), and SaSTP from *Streptococcus agalactiae* (Rantanen et al., 2007). The structures of these PPM homologs are nearly identical to that of the phosphatase core domain of human PP2C (Figures 6A and 6B), with the active site residues highly conserved (Figure 6C). Compared to human PP2C, two notable differences are the presence in these bacterial PPM homologs of a third metal ion and a loop above the active site (thought to regulate substrate binding and catalysis).

Despite recent progress, much remains to be learned about the molecular mechanism of PP2C. Unlike the PPP family, PP2C has a large number of isoforms encoded by different genes. The different isoforms have distinct sequences and domain organizations. These PP2C isoforms also exhibit distinct functions, expression patterns, and subcellular localization. How these isoforms are regulated during signaling remains largely unknown. The molecular determinants of substrate specificity for the various PP2C isoforms also remain to be elucidated. For example, in addition to the conserved PP2C phosphatase domain, PHLPP also contains an N-terminal PH domain and a leucine-rich repeat (LRR) domain (Brognard et al., 2007; Gao et al., 2005). PHLPP and PHLPP2 are thought to promote apoptosis and suppress tumor growth through dephosphorylation of distinct Akt isoforms (Brognard et al., 2007; Gao et al., 2005). But how the additional PH and LRR domains may contribute to the phosphastase activity and substrate specificity remains unclear.

FCP/SCP and Chronophin

In contrast to all other protein Ser/Thr phosphatases discussed herein, members of the FCP/SCP family rely on the aspartic acids of the sequence motif DxDxT/V for phosphatase activity. Another unusual feature is that FCP/SCP has only one primary substrate—the CTD of RNA polymerase II, which contains tandem repeats of the sequence YSPTSPS. There are eight putative CTD phosphatases in the human genome (Zhang et al., 2006). Both the level and the pattern of the CTD phosphorylation oscillate with cycles of transcription, with hypophosphorylation in the preinitiation complex and hyperphosphorylation during transcription elongation. Phosphorylated serine 5 (pSer5), the serine at the fifth position in the tandem repeat, is enriched at transcription initiation and early transcription elongation, whereas phosphorylation of the serine at the second position in the tandem repeat (pSer2) is favored during transcription elongation and through the end of transcription. Distinct patterns of phosphorylation in the CTD are assessed by regulatory proteins for binding (Fabrega et al., 2003; Glover-Cutter et al., 2008; Ho and Shuman, 1999) throughout transcription and constitute the so-called “CTD code” (Buratowski, 2003). Fcp1 is the main serine phosphatase for the CTD and can dephosphorylate both pSer2 and pSer5. Fcp1 from the fission yeast *Schizosaccharomyces pombe* favors pSer2 over pSer5 as the dephosphorylation substrate by a

factor of about 6-fold (Hausmann et al., 2004). In contrast, Scp1 exhibits little activity for pSer2 and prefers pSer5 by a factor of 70-fold (Zhang et al., 2006).

Structural analysis reveals that Scp1 forms an α/β fold, with a central five-stranded β sheet (Kamenski et al., 2004; Zhang et al., 2006) (Figure 7A). Despite a lack of detectable sequence homology, the Scp1 protein core resembles that of the phosphoserine phosphatase from *Methanococcus jannaschii* (Wang et al., 2001), β-phosphoglucomutase from *Lactococcus lactis* (Lahiri et al., 2003), the *Bacteroides* hexose phosphate phosphatase (Lu et al., 2008), and haloacid dehalogenase (HAD) from *Xanthobacter autotrophicus* (Ridder et al., 1999). The signature motif DxDxT is located C-terminal to the first β strand of the conserved core domain. A three-stranded β sheet, termed the insertion domain, immediately follows the DxDxT motif. The close proximity of the insertion domain to the catalytic aspartate residues suggests a potential role for the region in catalysis. Consistent with this notion, a pSer5-containing CTD peptide is bound to the cleft between the insertion domain and the core domain (Zhang et al., 2006) (Figure 7A). The recognition specificity is mainly provided by Mg^{2+}-mediated coordination of the phosphate group of pSer5 and interaction of the proline at the third position in the repeat (Pro3) with hydrophobic amino acids from the insertion domain (Figure 7A, right panel). This mode of binding provides a plausible explanation for why Scp1 favors pSer5 over pSer2.

Fcp1 forms a Y-shaped structure, with the conserved FCPH domain as the base and bottom stem (Ghosh et al., 2008) (Figure 7B). The single BRCT domain forms one arm, and the insertion domain, which contains four extra α helices compared to Scp1, constitutes the other arm. This architecture generates a deep canyon at the center of Fcp1, with the DxDxT motif and the bound Mg^{2+} ion at the base of the canyon. The structural arrangement excludes the possibility that the CTD peptide may bind to the same location in Fcp1 as in Scp1. Biochemical and structural analysis strongly argues that the canyon between the two arms of Fcp1 may accommodate the CTD substrate (Ghosh et al., 2008).

The catalytic mechanism of Fcp1/Scp1 may involve two sequential steps (Ghosh et al., 2008; Kamenski et al., 2004). First, an oxygen atom from the carboxylate group of the N-terminal aspartate in the DxDxT motif initiates a nucleophilic attack on the phosphorous atom of a pSer, forming an acylphosphate intermediate. Second, a water nucleophile, likely activated by the second aspartate in the DxDxT motif, attacks the phosphorous atom of the acylphosphate intermediate, resulting in the release of an inorganic phosphate. Mg^{2+} is thought to facilitate both steps of the reaction by neutralizing the negative charges of the phosphate group. It is important to note that the role of the metal ion (Mg^{2+}) in Fcp1/Scp1 is different from that in the PPP or PPM family, where the metal ions are directly involved in catalysis through the activation of a water nucleophile.

Chronophin, a member of the HAD family, is also an aspartate-based PSP (Gohla et al., 2005). Like FCP/SCP, it contains the signature sequence motif DxDxT and has a similar active site (PDB code 2CFR). Another striking similarity between FCP/SCP and chronophin is that they each have only one known substrate protein. Chronophin dephosphorylates pSer3 of cofilin, an important regulator of actin dynamics, leading to its activation (Gohla et al., 2005). The mechanisms by which chronophin recognizes and dephosphorylates cofilin remain to be elucidated.

Other Protein Ser/Thr Phosphatases

This review focuses on the PSPs for which structural information is available. Some of the other PSPs share considerable sequence similarity with the structurally characterized PSPs and are expected to exhibit similar structural features. For example, the catalytic subunits of PP4 and PP6 are closely related to the catalytic subunit of PP2A (Figure 1). Both PP4 and PP6 are essential PSPs in all eukaryotic species and are thought to regulate a diverse range of cellular functions independently of PP2A (Cohen et al., 2005; Kajino et al., 2006; Mi et al., 2009; Stefansson and Brautigan, 2007). In analogy to PP2A, the catalytic subunit of PP6 is thought to form a heterotrimeric holoenzyme, with a Sit4-associated protein (SAP) domain-containing scaffold subunit and an ankyrin repeat subunit that likely serves as the regulatory subunit (Stefansson et al., 2008). The catalytic subunit of PP4 also associates with its own regulatory subunits R1 and R2 to form distinct complexes (Cohen et al., 2005). In contrast to PP4 and PP6, PP7 appears to be unique to plants. Unlike other members of the PPP family, PP7 contains three insertions in its phosphatase domain, and the recombinant PP7 protein gained phosphatase activity only after cleavage of the longest insertion, suggesting an autoinhibitory role (Kutuzov et al., 1998). PP7 was shown to interact with Ca^{2+}-CaM, but in contrast to calcineurin, this interaction appears to inhibit the phosphatase activity of PP7 (Kutuzov et al., 2001).

There are also other unique PSPs. For example, protein phosphatases with kelch-like repeats (PPKLs) contain a C-terminal domain, a PP1-related Ser/Thr phosphatase domain, and a predicted N-terminal β-propeller domain comprising multiple kelch-like repeats (Kutuzov and Andreeva, 2002). The kelch repeats are likely involved in substrate recognition and interaction with other proteins. PPKLs have been identified in plants, green algae, and parasites (Moorhead et al., 2009). The function and molecular mechanisms of PPKLs remain largely uncharacterized.

Perspective

Many features of PSPs are different from those of PTPs. In contrast to PSPs, 99 of the 107 PTPs in the human genome are cysteine-based phosphatases, which are likely evolved from a common ancestor and share a similar structure for the phosphatase domain (Alonso et al., 2004). Consequently, the catalytic mechanisms of the metal-dependent PSPs—PPP and PPM—differ from those of the cysteine-based PTPs. Most PTPs contain one or more additional domains, which mediate interactions with other proteins and/or phospholipids. The single-chain, multidomain feature of PTPs, which may afford stringent specificity and tight regulation, contrasts with the multisubunit characteristic of most PSPs that likely generates greater substrate diversity and flexibility.

Phosphorylation primarily occurs on serine, threonine, and tyrosine residues in eukaryotic proteins. In contrast, prokaryotic proteins, such as those of the bacterial two-component

signaling systems, were thought to be mainly phosphorylated on histidine and aspartic acid residues. Recent phosphoproteomic analyses, however, have revealed widespread phosphorylation on serine, threonine, and tyrosine residues in many essential proteins of both Gram-positive and Gram-negative bacteria (Macek et al., 2007, 2008; Soufi et al., 2008). These observations, together with the finding that bacteria contain a number of PPM-like Ser/Thr phosphatases, suggest a critical role for serine/threonine/tyrosine phosphorylation and dephosphorylation in the prokaryotic life cycle. Discovery of Ser/Thr-specific and Tyr-specific kinases and phosphatases in bacteria and characterization of their activities are likely to reveal additional principles of bacterial signaling.

In the human genome, the number of genes encoding the catalytic subunit of protein Ser/Thr phosphatases is much smaller than the number of genes that encode protein Ser/Thr kinases. In this regard, how can the PSPs ensure specificity in cell signaling? The answer to this question appears to be straightforward for the PPP family of phosphatases. Through association with multiple regulatory subunits and other interacting proteins, each catalytic subunit gains the ability to form a large number of different holoenzymes, each with potentially distinct substrate specificity. This scenario is exemplified by PP1, which has more than 100 R subunits, and by PP2A, which has a unique tripartite organization. The scaffold and the catalytic subunits of PP2A are each encoded by two genes, giving rise to the α and β isoforms; the regulatory subunits of PP2A comprise four families, each also encoded by several genes. Together, the combination of these three subunits generates a large number of heterotrimeric PP2A holoenzymes, each of which could serve a distinct function. For example, the δ isoform of the B′ subunit is involved in dephosphorylation of Cdc25, which is required for its sequestration by the adaptor protein 14-3-3 in DNA-responsive checkpoints (Margolis et al., 2006). The release of Cdc25 would lead to activation of Cdc2/CyclinB and cell-cycle progression. In contrast, B′α specifically associates with and dephosphorylates the proto-oncogene c-Myc, resulting in its degradation (Arnold and Sears, 2006). In addition, other interacting proteins may help target PP2A to specific cellular locations and thus further enhance substrate specificity. Recent biochemical and structural characterization suggests that shugoshin-mediated recruitment of PP2A to the centromere might be solely intended for the dephosphorylation of cohesin, thereby preventing cleavage of cohesin by separase during meiosis I to prevent premature sister chromatid separation (Xu et al., 2009). Supporting this notion, shugoshin binding did not affect the dephosphorylation of peptide substrate (Xu et al., 2009). Identification of specific PP2A holoenzyme(s) or other PSPs and their targeting/interacting proteins in specific cellular processes is only beginning and will likely have a major impact on mechanistic understanding of cellular physiology.

Structure underlies function and its elucidation can reveal mechanism. Recent biochemical and structural investigation of protein Ser/Thr phosphatases has given considerable insight into the mechanisms that underlie their assembly, activation, catalysis, substrate recognition, and regulation. Despite these advances, comprehensive mechanistic understanding is far from complete. There are major unanswered questions for every family or subfamily of PSPs. What we know today likely represents only a small proportion of what is required to have a comprehensive understanding on the function and mechanisms of PSPs. First, structural and mechanistic characterization of the core components of PSPs remains preliminary. For example, despite the elegant work on the PP1-MYPT1 complex, we do not yet have a general understanding of the mechanisms by which the R subunits modulate the function and activity of the PP1 catalytic subunit. We also do not yet understand the mechanisms by which Ca^{2+}-CaM binds and activates calcineurin. We have no structural information on the B″ family of regulatory subunits or its complex with the PP2A core enzyme. Second, mechanistic information regarding the recognition and dephosphorylation of specific substrate protein is scant. In fact, the physiological substrate proteins for most PSPs remain to be identified, and this is a major current focus of the phosphatase field. At present, only a few structures of PSPs bound to substrate peptide have been elucidated; there is no structural information available for a PSP bound to a phosphoprotein substrate. Third, beyond the regulatory subunits, although PSPs interact with a large number of cellular and viral proteins, the mechanisms by which PSPs are regulated by these proteins remain largely enigmatic. For example, it is still unclear how the antiapoptosis protein α4 modulates the activity and specificity of PP2A through competition with the scaffold subunit (Kong et al., 2004; Prickett and Brautigan, 2004). However, the structure of Tap42, the α4 protein in *Saccharomyces cerevisiae*, has revealed that the protein has a TPR-like fold and allowed the identification of amino acids that are important for binding to PP2A (Yang et al., 2007). One of the few well-characterized examples of PSP interaction with viral proteins involves the small t antigen of SV40, which specifically inhibits the phosphatase activity of the PP2A core enzyme and interferes with recruitment of the regulatory subunits (Chen et al., 2007; Cho et al., 2007; Pallas et al., 1990; Walter et al., 1990).

At a more specific level, little is known about the regulation of phosphatase activity or specificity by the regulatory subunits of PSPs. Existing evidence suggests that some PSPs may exhibit phosphotyrosine phosphatase activity under some circumstances. For example, PP2A was found to have a basal level of phosphotyrosine phosphatase activity, and PTPA stimulated this activity by more than 10-fold while suppressing the phosphoserine/phosphothreonine phosphatase activity of PP2A (Cayla et al., 1990; Chao et al., 2006; Van Hoof et al., 1994). How PTPA accomplishes this remains to be investigated, although available evidence showed that PTPA and the PP2A core enzyme form a composite ATPase. Within this ATPase, ATP hydrolysis was required for the enhanced phosphortyrosine phosphatase activity of PP2A (Cayla et al., 1990; Chao et al., 2006). Other studies suggested that PTPA might function as a *cis-trans* prolyl isomerase with specificity for a conserved proline residue close to the active site of the PP2A catalytic domain (Jordens et al., 2006; Leulliot et al., 2006). Because the observed PTPA prolyl isomerase activity was stimulated by Mg^{2+}/ATP, it is possible that prolyl isomerization, fueled the ATP hydrolysis, may be responsible for the altered substrate specificity through conformational changes

in the active site. Regardless of the exact mechanism, these observations raise the question of whether PP2A can ever be a physiologically meaningful PTP in cells and, if so, under what cellular context. As PP2A is highly conserved from yeasts to mammals, it remains to be seen whether there are conserved phosphotyrosine substrate proteins for PP2A.

The regulation and function of PSPs may turn out to be more complex as regulatory subunits are characterized in greater detail. Nonetheless, the principal conclusion derived from such studies is unlikely to change, that is, both the activity and the phosphatase specificity of PSPs are subject to regulation in response to the formation of a wide variety of distinct complexes.

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