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THE STRUCTURE AND MECHANISM OF PROTEIN PHOSPHATASES: Insights into Catalysis and Regulation

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ABSTRACT

Eukaryotic protein phosphatases are structurally and functionally diverse enzymes that are represented by three distinct gene families. Two of these, the PPP and PPM families, dephosphorylate phosphoserine and phosphothreonine residues, whereas the protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine amino acids. A subfamily of the PTPs, the dual-specificity phosphatases, dephosphorylate all three phosphoamino acids. Within each family, the catalytic domains are highly conserved, with functional diversity endowed by regulatory domains and subunits. The protein Ser/Thr phosphatases are metalloenzymes and dephosphorylate their substrates in a single reaction step using a metal-activated nucleophilic water molecule. In contrast, the PTPs catalyze dephosphorylation by use of a cysteinyl-phosphate enzyme intermediate. The crystal structures of a number of protein phosphatases have been determined, enabling us to understand their catalytic mechanisms and the basis for substrate recognition and to begin to provide insights into molecular mechanisms of protein phosphatase regulation.

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PERSPECTIVES AND OVERVIEW

The regulation of cellular processes in response to external stimuli is fundamental to all living processes. It is now recognized that the reversible phosphorylation of proteins is an essential element of the numerous and varied mechanisms that have evolved to communicate these stimuli across a cell's surface and subsequently to cause changes in the activities and functions of intracellular proteins. Changes in the state of protein phosphorylation are regulated by two types of enzyme activities: those catalyzed by the protein kinases, which covalently attach a phosphate group to an amino acid side chain, and the reverse activity of protein phosphatases.

The attachment or removal of a phosphate group from a protein may have profound effects on that protein's activities and properties. For example, protein phosphorylation allows the regulation of enzymic activities by mediating allosteric conformational changes, or by directly blocking access to enzyme catalytic sites (64, 65). More recently it has been realized that an essential feature of signaling by protein phosphorylation is to modulate the formation of protein-protein interactions, a process that is coordinated by signaling modules such as src-homology domain 2 and phosphotyrosine binding domains, and by 14-3-3 proteins that recognize phosphotyrosine and phosphoserine residues, respectively, within sequence-specific contexts (89).

Nearly all aspects of cell life are regulated by reversible protein phosphorylation. Some examples include metabolic processes, gene regulation, cell cycle control, transport and secretory processes, the organization of the cytoskeleton, and cell adhesion. Reflecting the diversity and breadth of functions regulated by protein phosphorylation, a large proportion of intracellular proteins (30%) are subject to reversible protein phosphorylation, and it is perhaps not surprising that higher eukaryotes encode approximately 2000 and 1000 protein kinase and phosphatase genes, respectively, corresponding to 3% of their genomes (60).

Protein kinases and phosphatases belong to a small number of gene superfamilies, with each member of the gene superfamily being related by divergent evolution. This is a characteristic of most signal transduction proteins. The protein

Table 1 Nomenclature of protein phosphatases

PPP family	
Catalytic subunit	Regulatory subunits
PP1c	G_M, G_L, $M_{110} + M_{21}$, NIPP-1, RIPP-1, R110, p53BP2, L5, sds22, RB gene product, inhibitor-1, DARPP-32, inhibitor-2, splicing factor, kinesin-like protein, γ134.5 (Herpes simplex), R5
PP2Ac	A subunit (PR65) B subunit (PR55, PR72, PR61), eRF1, PTPA, SET, polyoma middle and small T antigens, SV40 small T antigen
PP2B	B-subunit, calmodulin, AKAP-79
Novel protein phosphatases of the PPP family	
PPP1:	PPY, Ppz1, Ppz2, Ppq1
PPP2A:	PP4, PP6, PPV 6A, sit4, Ppc1, Ppg1
PPP5:	PP5, RdgC
PPM family	
PP2C	
Arabidopsis ABI1	
Arabidopsis KAPP-1	
Pyruvate dehydrogenase phosphatase	
Bacillus subtilis SpoIIE phosphatase	
Protein–tyrosine phosphatase family	
Tyrosine-specific phosphatases	
Cytosolic, nonreceptor forms	
PTP1B, SHP-1, SHP-2	
Receptor-like, transmembrane forms	
CD45, RPTPμ, RPTPα	
Dual-specificity phosphatases	
CDC25	
Kinase-associated phosphatase	
MAP kinase phosphatase-1	

phosphatases are defined by three structurally distinct gene families (Table 1). The PPP and PPM families encode protein Ser/Thr phosphatases, whereas the protein tyrosine phosphatase (PTP) family includes both tyrosine-specific and dual-specificity phosphatases. Within each superfamily, considerable structural diversity is generated by the attachment of regulatory and targeting domains and/or subunits to the protein catalytic domain. Regulatory subunits and domains serve to localize the protein to particular subcellular localizations and modulate protein specificity, functions that are regulated by allosteric modification using second messengers and reversible protein phosphorylation.

The state of cellular protein phosphorylation at any time is a dynamic process that depends on the activities of protein kinases and protein phosphatases for their appropriate substrates. To understand these overall processes, we need to understand (*a*) the mechanisms by which protein kinases and protein phosphatases recognize their substrates, (*b*) their catalytic mechanisms, (*c*) how these enzymes are targeted to particular subcellular locations, and finally, (*d*) how these three latter processes are regulated. In this review we will describe recent progress made towards understanding these questions in relation to the protein phosphatases at a molecular level.

PROTEIN SERINE/THREONINE PHOSPHATASES OF THE PPP FAMILY

The protein Ser/Thr phosphatases PP1, PP2A, and PP2B of the PPP family, together with PP2C of the PPM family, account for the majority of the protein serine/threonine phosphatase activity in vivo (Table 1, above). While PP1, PP2A, and PP2B share a common catalytic domain of 280 residues, these enzymes are most divergent within their noncatalytic N- and C-termini and are distinguished by their associated regulatory subunits to form a diverse variety of holoenzymes.

PP1 is involved in controlling multiple cellular functions including glycogen metabolism, muscle contraction, cell cycle progression, neuronal activities, and the splicing of RNA. These different processes are regulated by distinct PP1 holoenzymes in which the same catalytic subunit (PP1c) is complexed to different regulatory and targeting subunits (42, 59). Similar to PP1, the diverse functions of PP2A in vivo result from the (at least) 15 distinct regulatory B-subunits that individually assemble with a core heterodimer consisting of PP2Ac and a 65-kDa A-subunit (106). Specific roles for each of the PP2A holoenzymes are not well delineated, although PP2A regulates processes that include metabolism, cell signaling and the cell cycle, and the control of telomerase activity. Mutations in the PP2A 55-kDa B-subunit lead to defects in cell division in *Drosophila* embryos (80), whereas the association of the small T-antigen of SV40 and polyoma viruses to the core heterodimer inhibits PP2A dephosphorylation of MEK and MAP kinase with resultant activation of the MAP kinase pathway (97). PP2B is characterized by its dependence on Ca^{2+} for activity. The enzyme consists of an A-subunit with an N-terminal catalytic domain and a C-terminal regulatory region containing binding sites for the PP2B B-subunit and calmodulin, and at the extreme C-terminus, an autoinhibitory sequence. Maximal Ca^{2+} stimulation of PP2B requires association of Ca^{2+} to both the B-subunit and to calmodulin. PP2B plays a crucial role in the Ca^{2+} signaling cascade of activated T-cells. Increases in T-cell Ca^{2+} concentrations promoted

by antigen presentation to the T-cell receptor stimulates PP2B to dephosphorylate the cytosolic subunit of the transcription factor NFAT1 (61). Dephosphorylated NFAT1 translocates into the nucleus (probably as a complex with PP2B, 94) where, in concert with other transcription factors, it induces expression of the IL-2 gene, one of the early genes in the T-cell activation pathway. Inhibition of this Ca^{2+} signaling cascade by immunosuppressant drugs suppresses T-cell activation.

PP1 and PP2A are specifically and potently inhibited by a variety of naturally occurring toxins such as okadaic acid, a diarrhetic shellfish poison and strong tumor promoter, and microcystin, a liver toxin produced by blue-green algae (77). Whereas PP2B is only poorly inhibited by the toxins that affect PP1 and PP2A, it was recently defined as the immunosuppressive target of FK506 and cyclosporin in association with their major cellular binding proteins, the *cis-trans* peptidyl prolyl isomerases FKBP12 and cyclophilin, respectively (74).

Novel protein phosphatases of the PPP family have been isolated using molecular cloning screening techniques (24) (Table 1, above). These proteins are expressed at low abundance, with some having targeting domains attached to the phosphatase catalytic domains in addition to regulatory subunits. One example, PP5, contains three tetratricopeptide repeat (TPR) domains fused to the N-terminus of the phosphatase domain (20). The TPR repeats are assumed to form protein-protein interactions. Recent data support this notion. For example, it has been found that the TPR domain of PP5 interacts with the TPR binding site of the protein chaperone hsp90 as a complex with PP5, hsp90, and the glucocorticoid receptor (GR) (95). This association is likely to be important in vivo, because 35% of the hsp90.GR is found in a complex with PP5. The TPR domains of PP5 may directly regulate the phosphatase activity, because proteolytic removal of the TPR domain of PP5 results in a 25-fold enhancement of activity (19). A similar enhancement of activity is observed with unsaturated fatty acids such as arachadonic acid and phosphatidyl inositol, which bind to the TPR domain, allosterically stimulating phosphatase activity (19).

Structure and Catalytic Mechanism

X-ray crystallographic structural data are available for PP1c (39, 40, 49) and PP2B (50, 69), and the crystallization of the PP2A B-subunit has been reported recently. Not surprisingly (given the high level of sequence similarity shared by members of the PPP family), PP1c and PP2B share a common catalytic domain structure and architecture.

The catalytic domain fold of PP1c and PP2B consists of a central β-sandwich of two mixed β-sheets surrounded on one side by seven α-helices and on the other by a subdomain comprising three α-helices and a three-stranded β-sheet

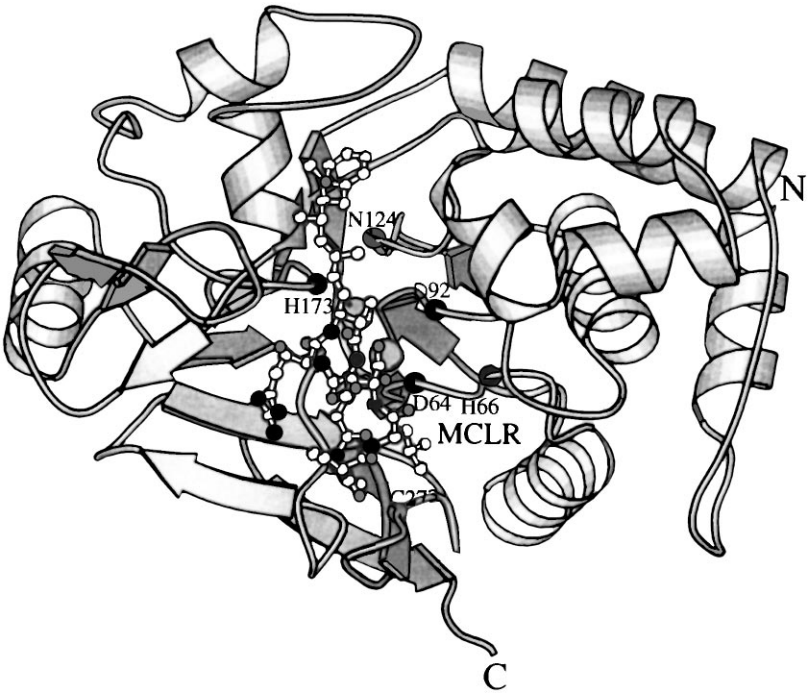

Figure 1 Protein phosphatase 1α in complex with microcystin LR (MCLR). MCLR interacts with the hydrophobic groove (via the Adda side chain), the metal sites (via a carboxylate group and a carbonyl oxygen of the toxin), and to Cys 273 (via the Mdha side chain). This structure, combined with the PP1-tungstate complex structure, reveals that microcystin inhibits the activity of PP1 by directly blocking substrate binding to the catalytic site.

(Figure 1). The interface of the three β-sheets at the top of the β-sandwich creates a shallow catalytic site channel. Amino acid residues present on loops emanating from β-strands of this central β-sandwich are responsible for coordinating a pair of metal ions to form a binuclear metal center.

Crystallographic data on PP1c (39, 49) and PP2B (50, 69) provided the first compelling information for the role of metal ions in the catalytic reaction of the PPP family. The identity of the two metal ions is slightly controversial. Proton-induced x-ray emission spectroscopy performed on PP1c crystals produced from protein expressed in *Eschericia coli* indicated that the metal ions were Fe^{2+} (or Fe^{3+}) and Mn^{2+} (39), whereas atomic absorption spectroscopy of bovine brain PP2B indicated a stoichiometric ratio of Zn^{2+} and iron (68, 111). There have also been conflicting reports concerning the iron oxidation states,

with both Fe^{2+} and Fe^{3+} observed (111). Native active PP2B is most likely to contain Fe^{2+}, and the time- and Ca^{2+}/calmodulin-dependent inactivation observed for PP2B results from oxidation of the Fe-Zn center that is prevented by superoxide dismutase in vivo and in vitro (108). Oxidation of the binuclear metal center and phosphatase inactivation may represent a novel mechanism for PP2B regulation by redox potential during oxidative stress (108).

The structures of PP1c with tungstate (39) and PP2B with phosphate (50) indicated that two oxygens of the oxyanion coordinate the metal ions. Two water molecules, one of which is a metal-bridging water molecule, contribute to the octahedral hexacoordination of the metal ions. The metal coordinating residues, aspartates, histidines, and asparagines, are invariant amongst all the PPP family members. These residues, together with Arg and His residues that interact with the phosphate group of the phosphorylated residue, occur within five conserved sequence motifs. These motifs are found in the sequences of other enzymes, including the purple acid phosphatase (99), that catalyze phosphoryl transfer reactions to water, suggestive of divergent evolution of these enzymes from an ancestral metallophosphoesterase (75). Consistent with roles in catalysis, mutation of these residues profoundly reduces catalytic rate (58, 113, 122).

Most data are consistent with the hypothesis that PPPs catalyze dephosphorylation in a single step with a metal-activated water molecule or hydroxide ion. The most compelling evidence for this notion is that the purple acid phosphatase, related to the PPPs at the catalytic site (99), catalyzes dephosphorylation with inversion of configuration of the oxygen geometry of the phosphate ion (85). This indicates that a phosphoryl-enzyme intermediate would not occur, consistent with the inability of PP2B to catalyze transphosphorylation reactions (79), and unlike the PTPs, the lack of detectable phosphoryl-enzyme reaction intermediates. The two metal-bound water molecules are within van der Waals distance of the phosphorus atom of phosphate bound to the catalytic site, one of which is the likely metal-activated nucleophile. Insight into the mechanism of the PPP-catalyzed dephosphorylation reaction has been provided by a recent study employing a phosphate monoester that is coordinated by two octahedral Co(III) centers which are also joined by two hydroxide bridges. This dinuclear Co(III) complex accelerates hydrolysis of the phosphate monoester at a greater rate than any other known chemical system. *Para*-nitrophenol phosphate, for example, is hydrolyzed with a rate constant of 445 M^{-1} s^{-1}. It was proposed that the mechanism of hydrolysis of *para*-nitrophenol phosphate involved a metal-bridging hydroxide that attacked the phosphate and displaced the phenolate. This hypothesis was supported by the finding that ^{18}O is incorporated into the product by labeling the bridging hydroxides with ^{18}OH. The bridging hydroxides do not exchange with solvent owing to the inertness of the binuclear Co(III) complex. In contrast, no label is incorporated into the phosphate

when the solvent is labeled with $^{18}OH_2$; hence the incoming nucleophile must be provided by a bridging hydroxide ion.

Further catalytic rate enhancement is provided by other catalytic site residues. For example, it is likely that the catalytic site Arg residues (Arg 96 and Arg 221 of PP1) stabilize the pentacoordinate transition state as loss of these residues dramatically reduces K_{cat} (58). In addition, His 125, which forms an ion pair with an invariant Asp residue, is ideally poised to donate a proton to the leaving group oxygen atom of the P-O scissile bond, a role consistent with the 10^6-fold loss of activity in PP1 (58, 113) and bacteriophage lambda phosphatase mutants of this residue (122). It is worth noting that the oxygen atom of phosphate hydrogen bonded to His 125 is directly opposite and in-line with the metal bridging water molecule.

Phosphatase Regulation

The C-terminal regions of PPP catalytic domains appear to be crucially important for communicating regulatory signals to the catalytic site. For instance, CDK-2 phosphorylation of a C-terminal Thr residue of PP1c inhibits PP1 in a cell-cycle–dependent manner (34, 110), an event that is an important process of cell cycle regulation. CDK2 phosphorylation and inhibition of PP1 prevents the reversal of CDK2-mediated phosphorylation of the retinoblastoma gene product, which must be phosphorylated for cells to enter the S-phase of the cell cycle (7). Methylation of the C-terminal Leu residue of PP2Ac moderately activates catalytic activity (43), and there are reports of tyrosine phosphorylation of the C-terminus of PP2Ac (18). The exact role of PP2A phosphorylation is not understood, but modulation of PP2Ac-regulatory subunit interactions is a possibility. The regulatory B-subunit of PP2B binds to a B-subunit binding helix of the A subunit, immediately C-terminal to the phosphatase catalytic domain (50, 69).

The mechanisms of inhibition of PP1c by microcystin LR (MCLR), and PP2B (both by its natural inhibitor, the autoinhibitory domain, and the FKBP12/FK506 complex) have been defined by the structure of the PP1-MCLR complex (49) and from two PP2B structures (50, 69). Microcystin LR is a complex cyclic heptapeptide that interacts with three distinct regions on the surface of PP1c (Figure 1, above). One of these involves a carboxylate and carbonyl group of the toxin that interacts with two of the metal-bound water molecules, hence blocking substrate binding directly. Other sites consist of a protein hydrophobic groove, and the $\beta12/\beta13$ loop where the Sγ atom of Cys 273 of PP1c forms a covalent bond to one of the side chains of the toxin, and the side chain of Tyr 272 packs against a leucine residue of the toxin. Interestingly, the conformation of MCLR does not change from its solution structure when in complex with PP1c (3), whereas the $\beta12/\beta13$ loop of PP1 undergoes a conformational

change that avoids steric conflict between Tyr 276 of PP1 and the Mdha side chain of MCLR (D Barford and M-P Egloff, unpublished information). The solution structure of another PP1/PP2A toxin, the heptapeptide motuporin, resembles that of MCLR, suggesting a similar model of interaction between PP1 and this inhibitor (3).

The role of the $\beta12/\beta13$ loop as a site of interaction with toxins was anticipated earlier from the finding that the substitution of Cys 269 of PP2A to Gly within the $\beta12/\beta13$ loop causes resistance to okadaic acid. The equivalent residue in PP1 is a Phe, and replacing this with a Cys enhances okadaic acid binding to PP1 (115). Other studies showed that mutation of Tyr 272 of the $\beta12/\beta13$ loop causes a dramatic loss of potency of okadaic acid, caliculyn A, and tautomyocin as well as microcystin and motuporin (114). These findings suggest that toxins of PP1 and PP2A, despite their dissimilar structures, interact with their targets through the $\beta12/\beta13$ loop. Significantly, the equivalent loop appears to play a role in interactions of PP2B with immunosuppressant-immunophilin complexes.

In the structure of the full-length PP2B holoenzyme, the autoinhibitory domain lies over the substrate binding channel of the catalytic domain in such a way that a Glu side-chain hydrogen bonds with two of the metal-bound water molecules (69). This interaction sterically hinders substrate binding and is reminiscent of the mechanism of inhibition of PP1 by MCLR (49).

Neither FKBP12 nor FK506 individually are capable of associating with PP2B; their interaction with PP2B requires a composite recognition surface of the binary complex. The quaternary complex of the PP2B holoenzyme with FKBP12 and FK506 indicates that the conformation of FKBP12 is nearly identical to the form in the binary complex of FKPB12 and FK506, with minor flexibilities observed in the His 87 to Ile 90 loop of FKBP12 (50, 69). The major site of interaction on PP2B is the base of the B-subunit binding helix of the A subunit, together with the B-subunit and a minor interaction with the $\beta12/\beta13$ loop of the catalytic site. FK506 is situated 25 Å from the catalytic site and cannot participate directly in phosphatase inhibition. FKBP12 sterically blocks access to the catalytic site for large macromolecules, although since its closest approach to the catalytic site is 10 Å, it does not prevent dephosphorylation of small molecule substrates such as *para*-nitrophenol phosphate. The conformation of neither microcystin nor the FKBP12-FK506 complex changes significantly on binding to their cellular targets PP1 (3) and PP2B (103), respectively, perhaps explaining their high affinity binding. It is likely that the mechanism of inhibition of PP2B by FKBP12-FK506 is similar to that for the cyclosporin/cyclophilin complex. Both these complexes compete for binding to PP2B, and mutations of the $\beta12/\beta13$ loop of yeast PP2B results in cyclosporin resistance (17). In the brain, PP2B forms a complex with

AKAP-79, an A-kinase anchoring protein, that is a noncompetitive inhibitor of PP2B (23).

Regulatory Subunits of PP1

Multiple proteins are substrates for PP1 and PP2A, the reversible phosphorylation of which are responsible for regulating diverse cellular functions. This seemingly paradoxical situation was resolved by the discovery that distinct forms of PP1 and PP2A holoenzymes occur in vivo, where the same catalytic subunit is complexed to different regulatory or targeting subunits (42, 59). For PP1, it has been shown that the latter class of subunit confer in-vivo substrate specificity by directing particular PP1 holoenzymes to a subcellular location and by enhancing or suppressing activity towards different substrates. For example, the M_{110} subunit, responsible for the association of PP1c with the myofibrils of skeletal muscle and smooth muscle, stimulates the activity of PP1 towards myosin light chain and suppresses activity towards glycogen phosphorylase (63). Similarly, G_L, which targets PP1 to liver glycogen, suppresses PP1 phosphorylase phosphatase activity (63). Besides defining specificity, regulatory subunits allow the activity of PP1c to be modulated by reversible protein phosphorylation and second messengers in response to extracellular stimuli. For example, the G_M subunit of PP1G is phosphorylated by PKA at Ser 67, an event that leads to the dissociation of PP1c from G_M and hence glycogen (27). Rho-mediated regulation of smooth muscle contraction and focal adhesions is exerted in part by Rho-GTP regulated protein kinase (ROK)-mediated phosphorylation of the M_{110} subunit, inactivating the myosin light-chain phosphatase activity of PP1M (67). Currently, $\sim$15 PP1-regulatory subunits are characterized (Table 1, above), but with the recent use of novel techniques such as microcystin-Sepharose affinity chromatography and the yeast–two hybrid system, this number will be greatly exceeded and at least 100 novel PP1-binding proteins have already been identified (16).

The binding of targeting subunits to the catalytic subunit of PP1 (PP1c) is mutually exclusive, suggesting one or more common or overlapping binding site(s), and it is a little surprising that there are no overall sequence similarities amongst most of the PP1-binding subunits. Recent experiments have begun to shed light on the molecular basis for the recognition of regulatory subunits by PP1c. Deletion mutagenesis of PP1 targeting subunits, together with synthetic peptides representing sequences within these subunits, has enabled the PP1c binding region of many PP1 regulatory subunits to be identified. PP1c is targeted to glycogen in liver and to glycogen particles and the sarcoplasmic reticulum in muscle by tissue-specific glycogen-binding subunits (G-subunits) (35, 84, 101). Comparison of G_M and G_L sequences identified three short, highly conserved regions, one being residues 63–86 of G_M (35), the region that encompasses

the site of PKA phosphorylation. A peptide corresponding to this sequence ($G_{M[63–75]}$ peptide) disrupts the interaction of PP1c with G_L, and also other PP1-binding subunits such as M_{110} and p53BP2 (40). This result implies that PP1c contains a recognition site for this peptide that overlaps with, or is identical to, its binding sites(s) for other regulatory subunits.

The recently determined crystal structure of PP1c in complex with the $G_{M[63–75]}$ peptide revealed a critical sequence motif important for the interaction of regulatory subunits with PP1c (40). Residues 64–69 of the $G_{M[63–75]}$ peptide are bound in an extended conformation to a hydrophobic channel within the C-terminal region of PP1c that is formed at the interface of the two β-sheets of the β-sandwich opposite to the catalytic site channel. Three residues of the peptide are incorporated into one of the PP1c β-sheets as a sixth β-strand parallel to the edge β-strand (β14). The predominant interactions between the peptide and PP1c involve Val 66 and Phe 68 of the peptide, which interact with hydrophobic residues of the channel together with Arg 64 and Arg 65 that form electrostatic interactions with acidic residues at the N-terminus of the channel.

The Val 66 and Phe 68 residues of the $G_{M[63–75]}$ peptide are critical in mediating the interactions of G_M and PP1c, because substitution of either of these residues for alanine abolishes (in the case of Phe) or severely reduces (in the case of Val) the ability of $G_{M[63–75]}$ peptides to bind PP1c. Interestingly, it was noted that the sequence RVSF that binds G_M to PP1c is invariant within G_L and related to a sequence (KVKF) within the N-terminal 38 residues of the M_{110} subunit. The $M_{110[1–38]}$ peptide mimics the M_{110} subunit by enhancing the PP1 activity towards myosin light chain (63); however, this activity is abolished if the VKF residues related to VSF of G_M are removed (40). Hence, a motif RVXF found in G_M and M_{110} is responsible for targeting these subunits to the same recognition site on PP1c in a mutually exclusive manner. Other data (reviewed in 40) indicated that relatively short fragments of PP1-binding subunits—for example, NIPP-1 (9, 105), p53BP2 (53), and an RNA splicing factor (55)—that retain the ability to bind to PP1 contain an R/KVXF motif. It is therefore likely that these proteins interact with PP1 in a similar manner to that of G_M. The realization that PP1 contains a binding site for the sequence RVXF provided a basis for understanding earlier published work concerning DARPP-32 and protein inhibitor-1, two proteins that become highly potent, nM, inhibitors of PP1c when phosphorylated on a Thr residue by PKA (Thr 35 of inhibitor-1, Thr 34 of DARPP-32). Previous studies had revealed that the N-terminal 8–38 residues of inhibitor-1 and DARPP-32 mimicked the full-length proteins in their ability to inhibit PP1c. Moreover, the identical sequence KIQF (similar to the R/KVXF motif) at the N-terminus of this sequence is necessary for mediating the inhibition of PP1 by these proteins. Loss of Ile 10 of the KIQF sequence of inhibitor-1

(2), or deletion of the motif, disrupts the inhibitory effects on PP1c by phosphoinhibitor-1 (41). A similar result was found on disrupting the equivalent residue (Ile 9) of DARPP-32 (33, 54). It was also discovered using biosensor studies that inhibitor-1 and DARPP-32 bind to PP1c in both their phosphorylated and nonphosphorylated states. Phosphorylation enhances binding affinity of both inhibitors by only two- to fourfold, whereas it causes a 10^6-fold increase in the inhibitory potency of DARPP-32. Moreover, although dephospho-inhibitor-1 binds to PP1, it is not a PP1c inhibitor (33, 41). These results were interpreted to indicate that inhibitor-1 and DARPP-32 bind to PP1c through two low-affinity sites, one that encompasses the sequence KIQF (similar to the RVXF motif of G_M and M_{110}), and another that includes the phosphorylated Thr residue and which presumably binds at the catalytic site. A peptide fragment of DARPP-32 (residues 8 to 38) completely antagonizes the inhibitory properties of phosphorylated DARPP-32 at a concentration of 15 μM (70), presumably because of competition between the peptide and protein at the RVxF binding site of PP1c that recognizes the DARPP-32 KIQF sequence.

The identification of a PP1 binding site for the RVxF motif of regulatory subunits (situated opposite to the catalytic site) explains the results that PP1c immobilized to microcystin-Sepharose affinity columns maintains an intact regulatory subunit binding site (83). It also explains how fragments of unphosphorylated DARPP-32 (residues 8–38) antagonize the inhibitory properties of phospho-DARPP-32 without affecting the inhibitory properties of microcystin, okadaic acid, or caliculyn A (70). An interaction between a relatively short amino acid sequence on PP1 binding subunits with PP1c explains not only the observation that multiple PP1-binding subunits exist, with no overall sequence similarities or shared domains, but also how the binding of targeting subunits to PP1 appears to be mutually exclusive. However, there is evidence that the RVXF motif binding site is not sufficient for high-affinity binding for all subunits, similarly to the situation for inhibitor-1 and DARPP-32, and a second low-affinity site is present on the G_M and M_{110} subunits (56, 63, 109).

The site of PKA phosphorylation on G_M, Ser 67, interacts with a Met residue and is in close proximity to a region of negative electrostatic potential on the protein surface. Phosphorylation of Ser 67 would introduce a bulky charged residue at this site that is energetically unfavorable, and this probably accounts for the dissociation of PP1c from phosphorylated G_M. A similar mechanism of control may also operate for other PP1-regulatory subunits. For example, NIPP-1, a nuclear RNA-binding protein, inhibits PP1 with an inhibitory constant of 1 pM (9). Phosphorylation of NIPP-1 by PKA and/or casein kinase 2 in vitro abolishes this inhibition (8) and these sites have been recently mapped to a central acidic region near to the RVXF-motif (104).

Model of PP1c-Phospho-Inhibitor 1 Complex

Using the knowledge that the KIQF sequence of inhibitor-1 and DARPP-32 binds to the RVXF binding site on PP1c, a plausible model of a complex of PP1c with phospho-inhibitor-1 has been constructed (Figure 2).

Secondary structure predictions of inhibitor 1 suggested that residues 9 to 14 and 23 to 31 adopt β-strand and α-helical conformations, respectively. The prediction of the sequence KIQF as a β-strand is consistent with our assumption that this region of inhibitor-1 adopts the same conformation as RVSF of the G_M peptide when bound to the VxF recognition site of PP1c. The residues RRPpTP encompassing the pThr 35 site were positioned within the catalytic site channel in an extended conformation, with the phosphate group of the pThr 35 occupying the phosphate binding site of the catalytic site. The four

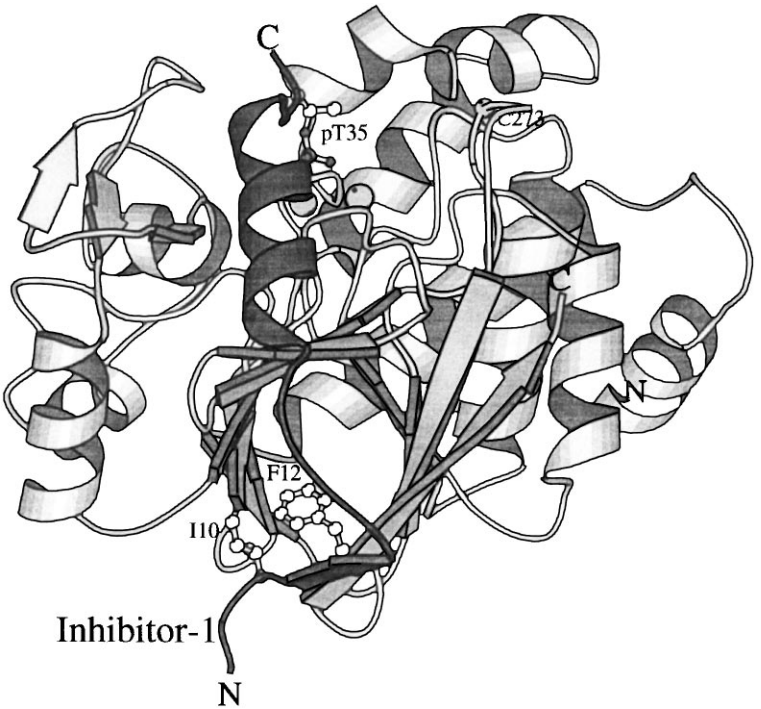

Figure 2 Structure of PP1c in complex with the residues 8 to 38 of phosphorylated inhibitor-1 (*dark shading*). The Ile 10 and Phe 12 residues interact at the (R/K)VXF motif of PP1, whereas the pThr-35 residue binds to the catalytic site.

consecutive Arg residues N-terminus to pThr 35 interact with Asp and Glu residues within an acidic groove of PP1c formed from the $\beta 7/\beta 8$ loop on one side and the $\beta 10/\beta 11$ loop and $\beta 11$ strand on the other, similar to that proposed by Goldberg et al (49) for their model of DARPP-32 bound to PP1c.

We propose that residues 20 to 30 of inhibitor-1 form an amphipathic helix that folds around the edge of the β-sandwich of PP1c. The N-terminus of this helix is disrupted by prolines at residues 19 and 23. Pro 19 and Pro 15 are probably responsible for introducing turns into the polypeptide chain that allows the β-strand encompassing the KIQF sequence (residues 9 to 14) to connect with the α-helix. The model of the phospho-inhibitor 1-PP1c complex is shown in Figure 2.

A final comment is that the mode of interaction between PP1c and a short peptide is similar to that observed in complexes of phosphotyrosine binding domains (PTB) (121) and PDZ domains (36) with their cognate peptide ligands. In these complexes, short peptides of 4-6 residues engage the protein by forming antiparallel hydrogen bonding interactions with edge β-strands that occur within a β-barrel. Formation of hydrogen-bonds between edge β-strands is observed at protein interfaces within numerous protein-protein complexes. These include (*a*) the streptococcal protein-G domain interaction with the C_H domain of IgG (31), (*b*) the Ras-binding domain of Raf kinase with Rap1A (86), and (*c*) the interaction of $p27^{Kip1}$ with Cdk2 within a ternary $p27^{Kip1}$-cyclin A-Cdk2 complex (91).

PROTEIN SERINE/THREONINE PHOSPHATASES OF THE PPM FAMILY

Protein phosphatases of the PPM family are present in both eukaryotes and prokaryotes whose defining member is PP2C. Within the PPM family, the PP2C domain occurs in numerous structural contexts that reflect functional diversity. For example, the PP2C domain of the *Arabidopsis* ABI1 gene is fused with EF hand motifs (73, 81) whereas in KAPP-1, a kinase interaction domain that associates with a phosphorylated receptor precedes the phosphatase domain (98). Other less closely related examples include the Ca^{2+} stimulated mitochondrial pyruvate dehydrogenase phosphatase, which contains a catalytic subunit sharing 22% sequence identity with mammalian PP2C (72), and the SpoIIE phosphatase of *B. subtilis*, which has ten membrane spanning regions preceding the PP2C-like catalytic domain (13, 37). A surprising homologue is a 300-residue region of yeast adenylyl cyclase, present immediately N-terminal to the cyclase catalytic domain, which shares sequence similarity with PP2C (66). This domain may function to mediate Ras-GTP activation of adenylyl cyclase activity and is not known to possess protein phosphatase activity.

In eukaryotes, one of the roles of PP2C is to reverse protein kinase cascades that become activated as a result of stress. For example, in mammalian hepatocytes, PP2C prevents inhibition of cholesterol and fatty acid biosynthesis resulting from elevated AMP/ATP ratios (82). Wip1, a novel PP2C enzyme, is induced in response to ionizing radiation in a p53-dependent manner, and ectopic expression of *wip1* in human cells suppressed colony formation (44). These results suggest that Wip1 might mediate growth inhibitory effects in response to DNA damage. The fission yeast enzyme negatively regulates the PBS2/HOG1-MAP kinase pathway that is activated in response to osmotic and heat shock (78). This may be achieved via dephosphorylation of a protein substrate downstream of the PBS2/HOG1-MAP kinase pathway (48). *Arabidopsis* PP2C is essential for transducing a signal by the hormone abscissic acid, leading to maintenance of seed dormancy, stomatal closure, and growth inhibition (73, 81). The role of PP2C-like protein phosphatases in regulating stress response pathways is also conserved in prokaryotes. The protein phosphatase SpoIIE controls the sporulation of *Bacillus subtilis* by dephosphorylating an antitranscription factor SpoIIAA, reversing the actions of the SpoIIAB protein kinase in a process that is governed by the ADP/ATP ratio (37).

The sequences of protein phosphatases of the PPM family share no similarity with those of the PPP family; however, the structures of these two families are strikingly similar (Figure 3) (26). Mammalian PP2C consists of two domains: an N-terminal catalytic domain with 6 α-helices and 11 β-strands, common to all members of the PP2C family, and a 90-residue C-terminal domain of 3 α-helices, characteristic of mammalian PP2Cs (see again Figure 3). The catalytic domain is dominated by a central, buried β-sandwich formed by the association of two antiparallel β-sheets, both of which are flanked by a pair of antiparallel α-helices inserted between the two central β-strands. The C-terminal domain is formed from three antiparallel α-helices remote from the catalytic site, suggesting a role in defining substrate specificity rather than catalysis.

At the catalytic site of PP2C, two Mn^{2+} ions within a binuclear metal center are coordinated by four invariant aspartate residues and a nonconserved Glu residue. These residues are situated at the top of a central β-sandwich. Six water molecules coordinate the two metal ions. One of these water molecules bridges the two metal ions and four form hydrogen bonds to a phosphate ion at the catalytic site. Dephosphorylation is probably catalyzed by metal-activated water molecules that act as nucleophiles and general acids in a similar mechanism to that proposed for the PPP family. Substitution of Asp residues of the yeast PP2C homologue, TPD1, and the *B. subtilis* SpoIIE phosphatase predicted by sequence alignments to be equivalent to metal coordinating Asps of human PP2C abolishes catalytic activity, supporting a role for metal ions in catalysis and the classification of SpoIIE as a PP2C-like protein phosphatase.

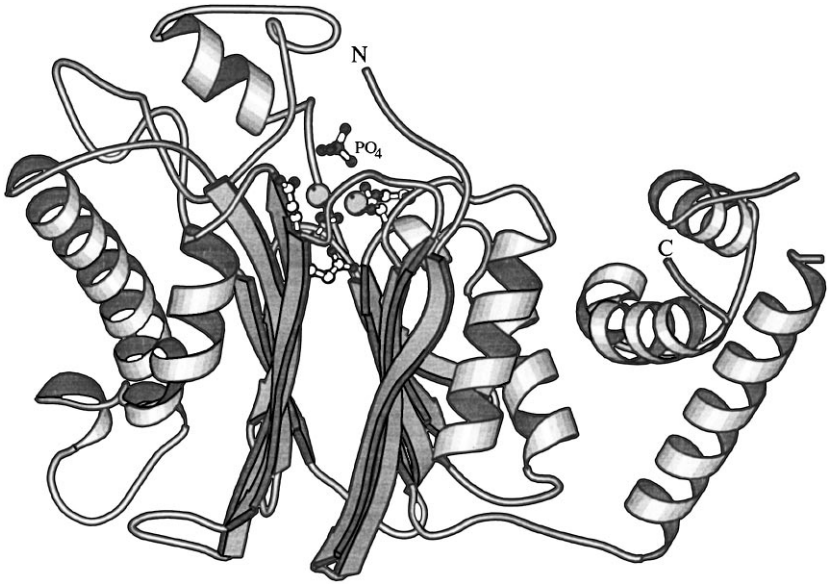

Figure 3 Human protein phosphatase 2C. The catalytic domain consists of a central β-sandwich surrounded by α-helices. The Mn^{2+} ions, *spheres*, are coordinated by Asp and Glu residues from the central β-sandwich structure.

PROTEIN TYROSINE PHOSPHATASES

The evolution of reversible tyrosine phosphorylation is linked to the development of multicellular organisms, being intimately associated with transmembrane signaling events. Numerous extracellular stimuli—for example hormones, growth factors, antigens, and cell-cell and cell-matrix interactions—activate receptor protein tyrosine kinases (PTKs) and/or receptor-associated soluble protein tyrosine kinases, leading to an increase in the levels of cellular tyrosine phosphorylation and the triggering of downstream signaling pathways. These pathways frequently involve the generation of second messengers, regulation of Ser/Thr phosphorylation, and G-protein activation. The importance of tyrosine phosphorylation in mediating intercellular communications is reflected in the increasing number and diversity of protein tyrosine kinases and phosphatases with an increase in eukaryotic complexity. Thus, multicellular organisms may encode approximately 500 PTKs, with perhaps a few hundred PTPs within their genomes. In contrast, yeasts encode no authentic PTKs and only two PTPs.

Similarly to the PTKs, diversity of the PTP family includes receptor-like transmembrane proteins and soluble cytosolic proteins (Table 1). Each PTP

contains a highly conserved catalytic domain of ~240 residues that shares high sequence similarity throughout the family. One of the hallmarks of the RPTPs is that most possess (with the exception of RPTPβ) two PTP domains arranged in tandem. The biological significance of this is not understood. For some, such as CD45, the membrane distal domain is inactive, and lacks many of the residues required for catalytic activity (6). Since the structures of PTP catalytic domains are highly conserved, the structural and functional diversity within the family is generated by noncatalytic regulatory and targeting domains attached to the N and C termini of the catalytic domain. Such domains function not only to regulate PTP catalytic activity but also to target the enzyme to particular subcellular locations (87). The net effect of these domains is to confer in vivo substrate specificity upon PTPs, and this is well exemplified by PTP1B and the SH2-domain containing PTPs, SHP-1, and SHP-2. The catalytic domain of PTP1B shares an average of 40% sequence identity with other members of the family and in vitro catalyzes the dephosphorylation of a wide spectrum of tyrosine phosphorylated peptides and proteins, although with some preference for pTyr residues immediately C-terminal to Asp and Glu containing sequences. Ectopic expression of the PTP1B catalytic domain within eukaryotic cells causes uniform reduction in the levels of tyrosine phosphorylation.

Moreover, so-called substrate trapping mutants of PTP1B (D181A) have been generated that inactivate the enzyme but allow formation of stable phosphatase-substrate complexes. When expressed in cells, the catalytic domain of PTP1B D181A associates with numerous tyrosine phosphorylated proteins (45). In contrast, expression of the full-length PTP1B D181A mutant, which contains a C-terminal endoplasmic reticulum targeting domain (46), restricts the association of PTP1B to only three proteins, one of which is the EGF receptor. The related SH2 domain-containing PTPs, SHP-1, and SHP-2, negatively and positively regulate signaling pathways downstream of activated cell-surface receptors, respectively (reviewed in 87). Within these proteins, the N-terminal SH2 domains serve the dual roles of targeting and regulation. Stimulation of receptor-associated PTKs and concomitant phosphorylation of receptor tyrosine residues leads to the engagement of pTyr residues within sequence-specific contexts, by the phosphatase SH2 domains. Hence the enzymes are recruited to their cellular substrates at the cell surface, and this is accompanied by a conformational change within the protein that activates PTP catalytic activity. Other noncatalytic domains of the non-transmembrane PTPs include domains with homology to cytoskeletal and lipid binding domains, SH3 domain-recognition polyproline sequences, and PEST sequences.

Receptor-like PTPs (RPTPs) possess the potential to communicate transmembrane signals via modulation of the activity of their intracellular catalytic domains and resultant change of cellular tyrosine phosphorylation. The regulation of cell adhesion may be an important function of one group of

four RPTPs that possess extracellular segments with fibronectin-type III and immunoglobulin-domains, hence sharing structural similarity to cell-adhesion molecules of the N-CAM and V-CAM families. Overexpression of the extracellular segments of RPTPμ and RPTPκ on the surface of insect cells causes homotypic interactions (14, 92). For RPTPμ, RPTPκ, and RPTPλ, an interaction with the cadheren/β-catenin complex has been demonstrated in vitro, consistent with the subcellular localization of these RPTPs to cell adherens junctions in vivo (15, 21, 47). It is likely that these RPTPs regulate the level of tyrosine phosphorylation within adherens junctions and the associated interaction of these junctions with the cytoskeleton.

A subfamily of the tyrosine-specific PTPs is termed the dual-specificity phosphatases (DSPs), so called because in vitro these enzymes catalyze the dephosphorylation of all three phospho-amino acids, although they show strong preferences for particular protein substrates in vitro and in vivo. Examples include the cell cycle regulators CDC25 and kinase associated phosphatase (KAP) and MAP kinase phosphatases. These enzymes are related to the PTPs by their possession of the conserved PTP signature motif, similarities of catalytic mechanism, and (it is now known) similarities in tertiary structure.

Structure

Protein tyrosine phosphatases and the dual-specificity phosphatases are characterized by the PTP signature motif (I/V)HCXAGXGR(S/T) containing the catalytically essential Cys and Arg residues. Within the PTPs and DSPs, the signature motif is situated at the center of the molecule, at the base of the catalytic site. The sequence forms the C-terminus of a β-strand, a loop connecting the β-strand with an α-helix, and the first turn of the α-helix (Figure 4).

The three-dimensional structures of all PTP catalytic domains known share a common architecture of a central, highly twisted β-sheet of nine β-strands with four central parallel β-strands flanked by antiparallel β-strands (5, 100). This sheet is surrounded by α-helices with four on one side and two on the opposite side. Additional secondary structural elements present N- and C-terminal to the conserved PTP domain are accommodated within this domain. For example, in common with the other eukaryotic PTPs, two N-terminal α-helices (α-1′ and α-2′) pack against α-5 and α-6 of PTP1B. The PTP signature motif, located at the base of the catalytic site cleft, is surrounded by four loops, three of which provide residues necessary for catalysis and substrate specificity. One of these loops, which forms part of the phosphotyrosine recognition region located N-terminus to the β-sheet, is critical in defining the specificity of the PTPs for phosphotyrosine (62).

The catalytic domains of the dual-specificity phosphatases VHR and KAP, despite sharing no sequence-similarity between themselves or with the PTPs,

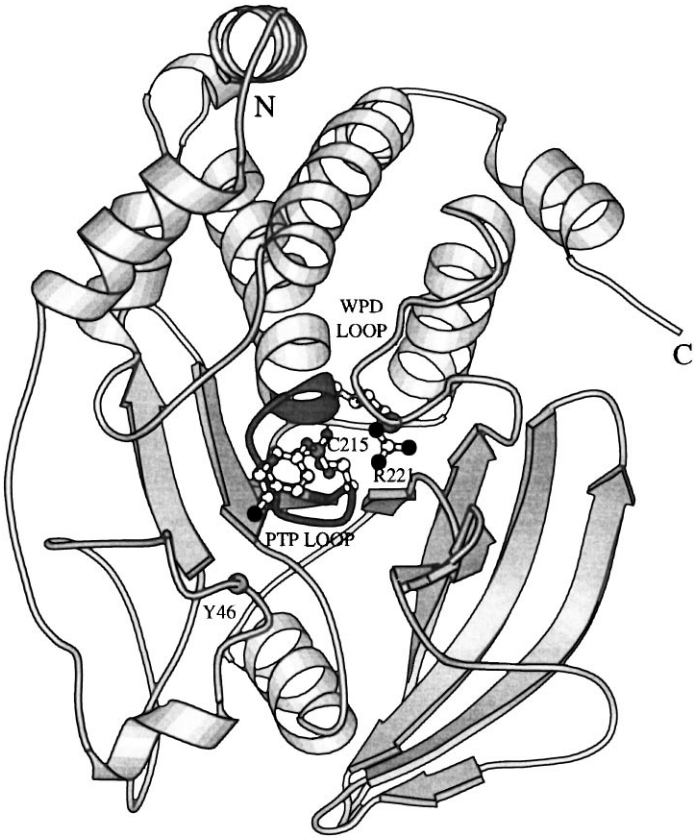

Figure 4 Structure of protein tyrosine phosphatase 1B. The PTP loop (*dark shading*) and WPD loop are indicated, as is Cys 215 and Arg 221 of the PTP loop and the position of the Cα-atom of Tyr 46 of the phosphotyrosine recognition loop.

reveal essentially the same core structural features as the PTPs, namely the central four-stranded parallel β-sheet surrounded on both sides by one and four α-helices (112; N Hanlon, M Groves, and D Barford, unpublished information). However, the DSPs appear to be a truncated version of the PTPs, and have only one of the antiparallel flanking β-strands and lack three of the antiparallel β-strands that flank one side of the β-sheet of the PTPs and which are instead replaced with either one (VHR) or two (KAP) short α-helices. This region of the catalytic domain of PTPs and DSPs has been referred to as the variable insert region. Also lacking within the DSPs is the phosphotyrosine recognition subdomain.

Catalytic Mechanism

Much is now understood concerning the mechanism of PTP catalysis and specificity for pTyr-containing peptides. The phosphate group of pTyr is coordinated by main-chain amide groups and the Arg side chain of the PTP motif so that the phosphorus atom is situated adjacent to the Sγ-atom of the catalytic Cys residue (62). Engagement of phosphopeptides to PTP1B promotes a major conformational change of one of the catalytic site loops (the WPD loop) consisting of residues 179 to 187 that shift by as much as 8 Å to close over the phenyl ring of pTyr and allow the side chain of Asp 181 to act as a general acid in the catalytic reaction. The Arg 221 side chain reorients to optimize salt-bridge interactions with the phosphate bound to the catalytic site. This shift is coupled to motion of the WPD loop via a hydrogen bond between NH2 of Arg 221 and the carbonyl oxygen of Pro 180, and hydrophobic interactions between the aliphatic moiety of Arg 221 and the side chain of Trp 179. These interactions and the hydrophobic packing between Phe 182 and the phenyl ring of pTyr stabilize the closed, catalytically competent conformation of the loop. The phosphotyrosine dephosphorylation reaction commences with nucleophilic attack by the Sγ-atom of the catalytic cysteine on the pTyr phosphorus atom. Cleavage of the scissile P-O bond is facilitated by protonation of the phenolic oxygen by Asp 181 with the consequent formation of a phospho-cysteine intermediate. This transient intermediate is hydrolyzed by an activated water molecule. Phosphoryl transfer reaction to a water molecule catalyzed by PTPs is highly specific because PTPs are unable to phosphorylate a range of primary alcohols and other phosphoryl-acceptors (Z-Y Zhang, personal communication). The structure of a PTP1B-tungstate complex suggests that in the presence of a phosphate, the WPD loop opens, allowing product release (5).

Numerous kinetic data support the reaction mechanism outlined above (see 29 for a review). Cysteinyl-phosphate intermediates have been trapped by rapid denaturation of PTPs and a dual-specificity phosphatase (VHR) during catalytic turnover (22, 52, 90, 120). Moreover, substitution of the catalytic Cys residue for a serine abolishes catalytic activity and the formation of a cysteinyl-phosphate intermediate (52). The nucleophilicity of the Cys residues results from its close proximity to main-chain amide groups and a hydrogen bond with the side chain of Ser 222 of the PTP signature motif, and has an unusually low pK_a of 4.6 (116). The catalytic Asp residue (Asp 181 of PTP1B) contributes to the basic limb of the pH activity profile, and its substitution to Ala causes a 10^5-fold reduction in k_{cat}, suggestive of a role as an acid catalyst (45, 119). This implies that Asp 181 is necessary for the first step of the reaction, namely cleavage of the pTyr P-O bond and intermediate formation, a notion consistent with the finding that Asp 181 mutants of PTP1B allow phosphorylated substrates to form stable complexes with the enzyme in vivo (45). The consequence of the

catalytic role played by the catalytic Asp residue (Asp 181 of PTP1B) is that the rate of hydrolysis of a range of aryl-phosphate esters is independent of the pK_a of the leaving-group residue (29).

The structures of PTP complexes with vanadate provided insights into the second step of the reaction, namely the hydrolysis of the cysteinyl-phosphate intermediate (28). Vanadate forms a covalent bond with the Sγ-atom of the nucleophilic Cys residue to produce a pentavalent trigonal bipyramidal configuration that is analogous to the transition state. The apical oxygen atom, which most closely resembles the attacking nucleophilic water molecule, forms a pair of hydrogen bonds to Asp 181 and to Gln 262. This observation led to the hypothesis that the role of Gln 262 is to position a water molecule for nucleophilic attack onto the cysteinyl-phosphate intermediate, and hence substitution of this residue for Ala should decrease the rate of cysteinyl-phosphate hydrolysis without affecting the rate of its formation. In this situation, the rate-limiting step becomes that of phosphocysteine hydrolysis, and hence the intermediate should accumulate. Direct visualization of this intermediate was achieved within PTP1B Q262A molecules by soaking crystals at 4°C in a large molar excess of *para*-nitrophenol phosphate (a pTyr analogue) and freezing the crystals at 100 K to trap, at steady state, the accumulation of the intermediate. The structure of the PTP1B-cysteinyl-phosphate intermediate demonstrates that a water-molecule hydrogen bonded to Asp 181 of the closed WPD loop was situated above the phosphate group of the cysteinyl-phosphate residue. However, the position of the water molecule was not ideal for in-line attack onto the phosphorus atom of the intermediate, being displaced from such a position by 1 Å. It is likely that this accounts for the reduced rate of hydrolysis of the PTP1B mutant, and the shift of this water molecule is probably caused by the loss of hydrogen bonding to the Gln 262 residue. Each step of the reaction pathway catalyzed by the PTPs may now be delineated in detail. A schematic is outlined in Figure 5 (ADB Pannifer and D Barford, unpublished MS).

The reactions catalyzed by PTPs share many features in common with that of GTP hydrolysis by the GTPases. For example, the role of Gln 262 in positioning a water molecule for nucleophilic attack onto the phosphocysteine intermediate of PTP1B is reminiscent of the Gln residue at the catalytic site of most GTPases, including the Ras family and G_α-subunits of the heterotrimeric G-proteins. Mutation of the catalytic site Gln 61 residue in Ras causes cellular transformation (4) and a 10-fold rate-reduction of GTP hydrolysis (12, 30). Similarly, mutations of the equivalent Gln residue within the catalytic sites of $G_{\alpha s}$ and $G_{i\alpha}$ reduces the intrinsic rate of GTP hydrolysis and are associated with thyroid and pituitary tumors (71, 76). Crystal structures of complexes of $G_{\alpha t}$ and $G_{i\alpha}$ with GDP, Mg^{2+} and AlF^{4-} (25, 96) and a recent structure of a Ras-RasGAP, GDP, Mg^{2+}, AlF^3 complex (93) have revealed the active

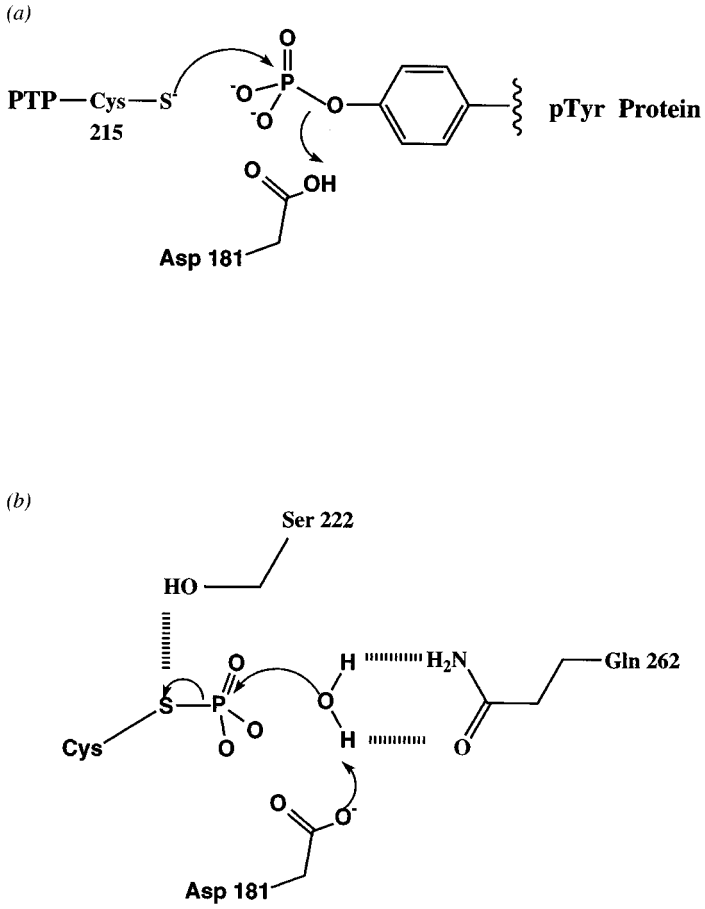

Figure 5 Schematic of the reaction mechanism catalyzed by PTP1B. (*a*) Formation of the cysteinyl-phosphate intermediate. (*b*) Hydrolysis of the cysteinyl-phosphate intermediate.

site conformations of these enzymes and the mechanisms of GTP hydrolysis. Gln 61 (and its homologue in the heterotrimeric G-proteins) plays a dual role in catalyzing GTP hydrolysis. First, it coordinates the attacking water molecule and positions it optimally for in-line approach onto the GTP-γ-phosphate. Second, it forms a hydrogen bond with the γ-phosphate oxygen atom as it passes through the transition state, stabilizing the pentavalent phosphorus transition state. In PTP1B, Gln 262 also coordinates the attacking water molecule to position it for in-line attack, although, unlike GTPase, Gln 262 does not

hydrogen-bond to the oxygens of the cysteinyl phosphate. In addition to a common Gln residue, both PTPs and GTPases use an essential Arg residue that coordinates and stabilizes the pentavalent phosphorus intermediate (25, 93, 96).

Substrate Specificity

PTPs are absolutely specific for pTyr-containing proteins, being unable to dephosphorylate pSer and pThr proteins (102). Hydrolysis of small-molecule phosphate monoesters such as free pSer and pThr proceeds at a measurable (although extremely low) rate, some 10^5-fold lower than that of pTyr hydrolysis. Amino acids N- and C-terminal to the pTyr residue confer additional binding affinity, and for PTP1B and a number of other PTPs, a preference is displayed for peptides with acidic residues preceding the pTyr residue and C-terminal hydrophobic residues (6, 117, 118). The molecular basis for substrate specificity results from the dimensions of the catalytic site cleft, which measures 9 Å from its base, where the nucleophilic Cys residue is positioned, to its entrance. A key structural component of the catalytic cleft is provided by the phosphotyrosine recognition loop formed from a conserved sequence KNRY (residues 43–46 of PTP1B). The Tyr residue of this motif packs against the phenyl ring of a pTyr substrate and is critical in defining the depth of the catalytic site cleft (62) (Figure 4, above). Other nonpolar residues present within the pTyr recognition loop, the WPD and PTP loops interact with the phenyl ring. An Asp residue present within the pTyr recognition loop forms a bifurcated hydrogen bond to the main-chain amide groups of the pTyr and P+1 residues of the peptide. This forces the pTyr residue to adopt a helical conformation and insert into the catalytic site cleft. Acidic residues present N-terminal to the pTyr residue interact with basic residues on the phosphatase surface, explaining the preference of this enzyme for peptides with acidic residues in these positions. The significance of the depth of the catalytic site cleft as a determinant of substrate specificity was elegantly demonstrated in a study showing that the *Yersinia* PTP was capable of dephosphorylating straight-chain peptide-bound aliphatic phosphates of the general structure: $(Glu)_4$-NH-$(CH_2)_n$-PO_3. The most efficient substrate is one with seven methylenes, that is, exactly as long as a tyrosine residue (38).

The DSPs differ from the PTPs because of their ability to dephosphorylate pSer and pThr proteins. A comparison of VHR with PTP1B and the *Yersinia* PTP revealed the expected similarities in the structure of the PTP motif at the base of the catalytic site (112). An Asp residue (Asp 92) is equivalent to the catalytic Asps of PTPs within both the three-dimensional structure and approximately 30 residues N-terminal to the PTP motif. The catalytic site of KAP shows more similarities to the PTPs than does VHR (N Hanlon, M Groves, D Barford, unpublished information). For instance, in KAP, as in the PTPs, a Glu residue forms an ion-pair with the catalytic site Arg residue,

and an equivalent Gln residue to the nucleophilic water coordinating Gln residue of PTPs (Gln 262 of PTP1B) is present. The most significant difference between the PTPs and DSPs is the absence of the pTyr recognition domain within the DSPs, the consequence of which is to produce a much shallower and open catalytic site with a depth of 6 Å, permitting the hydrolysis of the shorter pSer and pThr residues.

Regulation

PTPs are efficient catalysts, with constitutive activity of these proteins causing the rapid dephosphorylation of intracellular pTyr residues and consequent disruption of signal transduction pathways. Interestingly, this is a mechanism exploited by *Yersinia* bacteria, which inject proteins encoded by the Yop genes, including the YopH PTP, into macrophages, hence preventing immune-directed bacterial phagocytosis and leading to diseases such as the bubonic plague and tuberculosis (51, 11).

The isolated catalytic domains of most PTPs demonstrate activity, hence PTP regulation—at least in part—requires inhibition of this activity. Such a role may be played by the regulatory domains attached to the PTP catalytic domains. For the SH2 domain-containing PTPs, SHP-1 and SHP-2, numerous data support the notion that the N-terminal SH2 domains inhibit catalytic activity, most likely by binding to the PTP domain and blocking substrate access to the catalytic site. Engagement of phosphorylated peptides by the SH2 domains releases this inhibition with concomitant activation of the PTP's activity.

Structural support for such a mechanism of control came unexpectedly with the crystal structure of the membrane proximal domain (domain 1) of RPTPα (10). This structure revealed that the molecule was a homodimer in two independent crystal forms (Figure 6).

The biological significance of this structure lay in the observation that the catalytic site of each subunit was sterically blocked by the insertion of a wedge connecting two helices, $\alpha 1'$ and $\alpha 2'$, within a helix-turn-helix segment immediately preceding the PTP catalytic domain from the opposite subunit of the dimer. Residues of this wedge directly interact with the equivalent catalytic site residues which were part of the phosphopeptide binding site of PTP1B. Moreover, the WPD loop is stabilized in the open conformation and is sterically prevented from adopting the catalytically closed conformer. The overall tertiary structure of RPTPα is very similar to that of PTP1B; however, two differences in tertiary structure between these two proteins are significant in promoting the dimeric structure that is observed for RPTPα. One is a two-residue insertion within the wedge connecting $\alpha 1'$ with $\alpha 2'$ of RPTPα. These two residues participate at the dimeric interface of RPTPα and insert into the catalytic site. The second is a small β-sheet formed by βx at the immediate

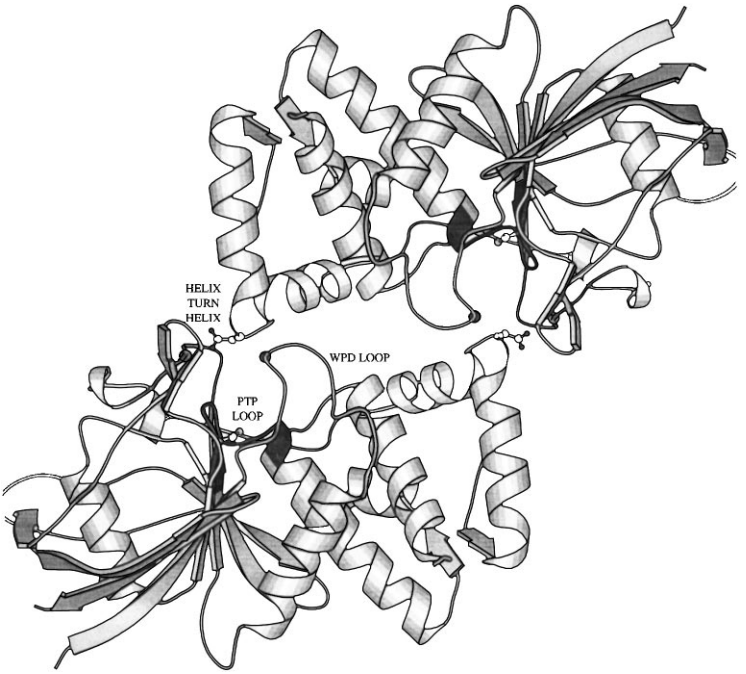

Figure 6 Domain 1 of receptor protein tyrosine phosphatase α. An example of a receptor-like PTP. In two independent crystal forms, the protein forms a homodimer such that the catalytic site (PTP loop) of each molecule is blocked by a wedge within a helix-turn-helix segment. This inhibits the enzyme by preventing substrate binding. The reason is that Asp 227 of one subunit interacts with the pTyr recognition loop of the opposite subunit and the WPD loop containing the catalytic Asp residue is restrained in the open, inactive conformation.

N-terminus of the $\alpha 1'$ helix and βy, C-terminus to the $\alpha 5$ helix. This sheet, absent from PTP1B, is important in stabilizing the conformation of the helix-turn-helix segment. Similarities in sequence between RPTPα and other RPTPs not shared with the cytosolic PTPs suggest that these two tertiary features will be conserved within the RPTP family. It is therefore possible, as proposed by Bilwes et al (10), that other RPTPs may adopt a similar quaternary structure.

The notion that dimerization of RPTPs may provide a mechanism for the modulation of the catalytic activity of RPTPs provided an attractive explanation of earlier data concerning the signaling properties of an EGF receptor-CD45 chimera. Ablation of expression of CD45, the prototypic RPTP, from T- or B-cells disrupts the normal signaling responses to engagement of antigen receptor. However, signaling can be restored by expression of membrane-targeted

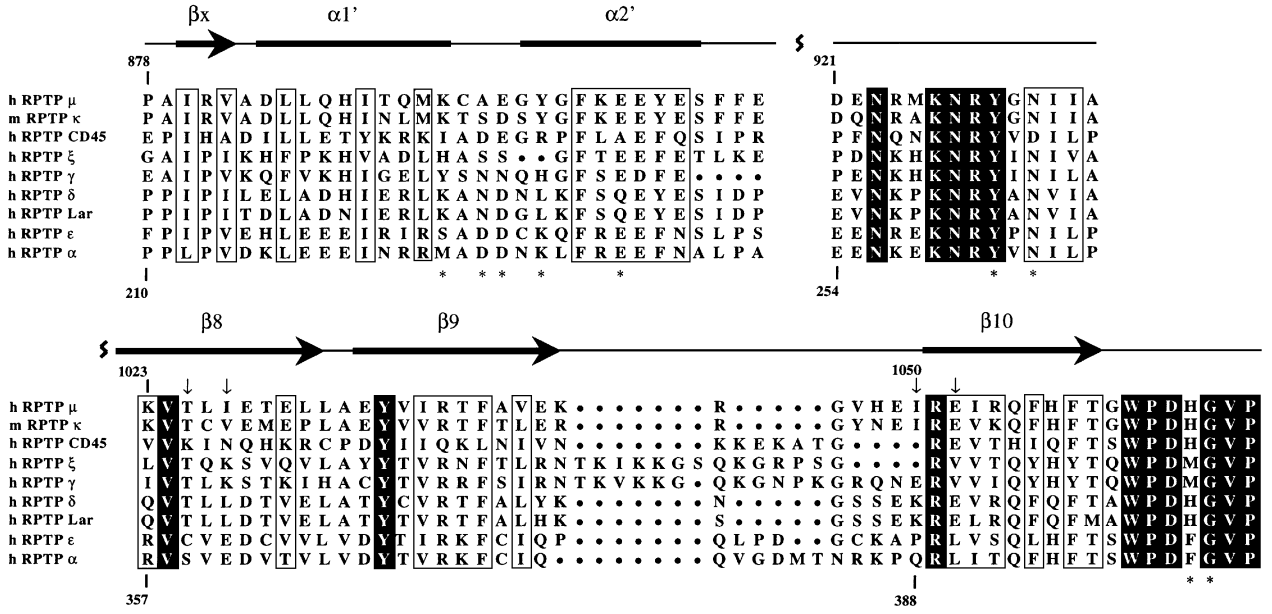

Figure 7 Sequence alignment of representative RPTP D1s in the regions that form the dimer interfaces of RPTPα and RPTPμ. *Top left*, βx-helix-turn-helix segment. *Top right*, residues of the pTyr recognition loop of the catalytic site. *Bottom*, residues from β8 to the WPD loop of the catalytic site. Residues of RPTPμ D1 and RPTPα D1 that form interactions at their respective dimer interfaces are indicated with vertical arrows (*top*) and stars (*bottom*), respectively. The residues that form the dimer interface of RPTPμ D1 are poorly conserved throughout the family, whereas residues of the RPTPα D1 interface are poorly conserved within the helix-turn-helix segment, but well conserved within the catalytic site.

constructs containing the catalytic domain of CD45. For example, a chimeric molecule where the extracellular and transmembrane segments of CD45 were replaced with those of the EGF receptor was able to restore signaling in the absence, but not in the presence, of EGF (32). In other words, EGF, the extracellular ligand of the chimeric molecule, blocked signaling from the phosphatase domain. It was proposed that in the presence of growth factor, dimerization of the extracellular segment of the chimera is induced that causes a trans-membrane signal resulting in dimerization of the PTP catalytic domain with concomitant inhibition of PTP activity. Interestingly, the sequence of the helix-turn-helix segment of CD45 is highly conserved with that of RPTPα, and therefore it is reasonable to suggest that CD45 may dimerize similarly to RPTPα.

For RPTPs with lower sequence similarities to RPTPα, dimerization involving the helix-turn-helix and catalytic sites would appear to be less likely. This assumption is based on the observation that residues of the wedge, which are variable amongst more distantly related RPTPs, interact with invariant catalytic site residues within the RPTPα dimer (Figure 7).

Direct support for this notion is provided by the crystal structure of RPTPμ D1 (57). RPTPμ D1 is a homodimer; however, although the tertiary structures of RPTPα D1 and RPTPμ D1 are very similar, the quaternary structures of these two proteins are different. Neither the catalytic site nor the N-terminal helix-turn-helix segment of RPTPμ D1 participates in protein-protein interactions. The catalytic site of RPTPμ D1 is unhindered and adopts an open conformation similar to that of the cytosolic PTP, PTP1B. The dimer interface of RPTPμ involved residues from β8 and β10 of the variable insert region.

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