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PHOSPHORYLASE PHOSPHATASE: NEW HORIZONS FOR AN OLD ENZYME

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TABLE OF CONTENTS

1. ABSTRACT

Protein phosphatase-1, originally studied as phosphorylase phosphatase, is one of the major ser/thr protein phosphatases. It has a long history and a complex enzymology. It consists of a catalytic subunit of 37 kDa, which is bound to a number of different regulatory or targeting subunits. These are believed to restrict its activity to its immediate microenvironment and thus define its specificity, as well as acting to regulate phosphatase activity. The existence of multiple protein phosphatase-1 binding proteins provides the mechanism whereby phosphatase-1 activity can be involved in a diverse range of cellular functions, and reflects a novel strategy for its evolutionary development.

2. INTRODUCTION

Protein phosphatase-1 (phosphorylase phosphatase), the prototype for the ser/thr phosphatases, has been studied since 1943 as the enzyme responsible for the conversion of phosphorylase *a* to phosphorylase *b* (1). It was originally named PR enzyme since its mode of action was thought at first to be a prosthetic group removal (phosphorylase *a* is active in the absence of 5'-AMP, while phosphorylase *b* requires 5'-AMP for its activity), and later the proteolytic cleavage of phosphorylase *a* (2). The discovery that PR enzyme was a phosphatase came at the same time (1955) as the discovery of phosphorylase kinase (3,4), findings which marked the beginning of the era of the study of protein phosphorylation/dephosphorylation as a regulatory mechanism. Investigation of phosphorylase phosphatase in the following three decades focused on defining its enzymology and role in glycogen metabolism (reviewed in 5-8; for more recent reviews, see 9-13). Progress in the attempts to isolate and characterize phosphorylase phosphatase activity was slow. The enzyme proved to be an elusive target, and presented a puzzling behavior in that partially purified phosphorylase phosphatase preparations exhibited a variety of molecular weight forms in different laboratories, and was extremely unstable (5,13). At the time, a key question was whether the phosphatase activities which acted on the three phospho-enzyme systems of glycogen metabolism (phosphorylase, glycogen synthase and phosphorylase kinase) were separate entities or whether they represented distinct proteins. Attempts to define this question also provided some puzzling findings, in that preparations with different ratios of phosphorylase phosphatase and glycogen synthase phosphatase activities were encountered. Today these earlier difficulties can be understood in terms of the concept that the enzyme system consists of a catalytic subunit that exists in complexes with a variety of targeting or regulatory subunits, generating multiple forms of different sizes and enzymatic behavior. The study of the enzymology of this exquisitely complex enzyme is still incomplete and has taxed the ingenuity and imagination of many investigators. Phosphorylase phosphatase, later

renamed protein phosphatase-1 (8), continues to bring surprises as well as a range of new questions regarding its cellular function.

In this review we will focus on the enzymology of protein phosphatase-1, in particular that of the catalytic subunit, from the perspective of work performed in our laboratory during the past twenty five years, as well as on currently unresolved questions regarding its properties and regulation. There have been many laboratories which have made important contributions to the study of this enzyme over its 55 year history, and there are a number of reviews which cover the general topic of the ser/thr protein phosphatases (5-14). Thus, in this summary we ask indulgence for the failure to cite all pertinent references.

3. BRIEF HISTORICAL BACKGROUND

Some of the earlier aspects of the study of phosphorylase phosphatase are summarized to provide a background for the discussion of more recent findings of the properties of protein phosphatase-1.

3.1. Isolation of the catalytic subunit and the recognition that it is part of holoenzyme

The key steps that led to the beginning of our understanding of the enzymology phosphorylase phosphatase came with the isolation of the catalytic subunits of liver and muscle phosphorylase phosphatase (15,16). Underlying this success was discovery that treatment of tissue extracts with denaturants (alcohol or urea) led to the release of a 35 kDa catalytic subunit which could be readily isolated (17). (For the remainder of this review, we will refer to the catalytic subunit simply as PP1.) This treatment released the catalytic subunit from its regulatory subunits, so that the enzyme activity now behaved in a monodisperse manner and was amenable to isolation by conventional chromatographic methods. The procedure that we used involved ammonium sulfate precipitation, followed by treatment with five volumes of 95% ethanol at room temperature. Following this, the phosphorylase phosphatase activity could be extracted from the pellet of denatured proteins. [As might be expected, this brutal procedure was not developed by design, and was discovered quite serendipitously through a misinterpretation of instructions on the use of alcohol precipitation by a summer student!] It became quite clear that while phosphorylase *a* appeared to be the best substrate for the catalytic subunit, both glycogen synthase and phosphorylase kinase were also dephosphorylated. Later studies (8) showed that this enzyme was relatively nonspecific (as compared for example to the protein kinases). The 35 kDa catalytic subunit was derived from a 37 kDa form by proteolytic cleavage near the C-terminus (8,13). In the course of the isolation of the rabbit muscle catalytic subunit we also isolated a second catalytic subunit of similar size, which differed in its ability to act on p-nitrophenyl phosphate (18). This later turned out to be the catalytic subunit of the phosphatase activity designated as type 2A phosphatase (8,9).

3.2. Activation of phosphorylase phosphatase and the discovery of inhibitory proteins

We discovered that ethanol or trypsin treatment of tissue extracts led to large increases in phosphorylase phosphatase activity, concomitant with the reduction in apparent size to the free catalytic subunit (17). These findings could be rationalized by the removal of an inhibitor protein(s) which were complexed with the catalytic subunit, and led to the discovery of the heat-stable PP1 inhibitor proteins of (19). The findings for the existence of heat-stable, trypsin-sensitive protein inhibitors of PP1 (17,19) played an important role in unraveling the enzymology of PP1 from two perspectives, the role of inhibitor proteins in regulating phosphatase activity, and the existence of holoenzyme forms. Subsequent studies (20) identified two such inhibitors: inhibitor-1 and inhibitor-2, which have been extensively studied (for reviews, see 8-10). Inhibitor-1 and inhibitor-2 are small proteins (165 and 204 a.a. residues, respectively) with estimated molecular masses of 23 and 19 kDa (8). However, both proteins, consistent with their heat-stability, appear to have little ordered structure and behave with anomalous high apparent molecular masses on both SDS-PAGE and gel filtration (8). Inhibitor-1 is unusual in that is only inhibitory when phosphorylated at a threonine residue by cAMP-dependent kinase (8).

3.3. Isolation of the holoenzyme forms of PP1

The concept that we proposed (17) for a holoenzyme complex of the catalytic subunit with regulatory subunits was supported by the findings that phosphorylase phosphatase activity generally exhibited high molecular weight characteristics on gel filtration, but could readily be reduced to a low molecular weight form by proteolysis, urea or ethanol treatment (21,22). Thus, a great deal of effort went into the isolation of the holoenzyme form of phosphorylase phosphatase. Our own attempts to isolate a high molecular weight form of phosphorylase phosphatase activity from rabbit muscle extracts led to the isolation of a two subunit enzyme which represented a holoenzyme form of PP2A (23), first isolated as a myosin light chain phosphatase by Pato and Adelstein (24). However, studies of an inactive form of phosphorylase phosphatase that could be activated by ATP ("ATP-Mg dependent phosphorylase phosphatase"), and that had been studied by Wilfried Merlevede for many years, led to its isolation and the surprising finding that it consisted of a 1:1 complex of the 37 kDa catalytic subunit and inhibitor-2 (6,10,25). The effect of ATP was due to the presence of a novel protein kinase which mediated the activation of the complex (25). The protein kinase "F_A" was found to be identical to a protein kinase which phosphorylated glycogen synthase (GSK-3). There have been extensive studies both of the isolated and reconstituted complex (reviewed in 6-8,10,14). Inhibitor-2 functions as an inhibitor of free PP1, *i.e.*, it causes an immediate inhibition of phosphorylase phosphatase activity. However, a slower time-dependent reaction takes place, that results in the conversion of PP1 to an inactive form that can be stimulated by the addition of Mn^{2+} when it is removed from the inactive complex. This inactivation reaction is readily monitored by trypsin treatment to

remove inhibitor-2, followed by the assay of phosphorylase phosphatase activity in the absence and presence of Mn^{2+} (10, 14, 26).

The complex exhibits some unusual properties in terms of its regulation by F_A/GSK-3 (7,10,14). Phosphorylation of the inactive PP1/inhibitor-2 heterodimer by GSK-3 (glycogen synthase kinase-3) at Thr72 does not result in activation, but allows access of a divalent metal ion to PP1. This activates PP1, thus permitting an intramolecular autodephosphorylation of inhibitor-2. It is during this autodephosphorylation that PP1 is converted to a fully active enzyme that is not dependent on metal ions. The activation is transient, as there is a slow conversion of PP1 back to the inactive form in a reaction that is similar to the effect of inhibitor-2 when added to free PP1. Thus, the phosphorylation of this complex is unusual in that it only results in a transient activation of PP1 that is not correlated with the phosphorylation state of inhibitor-2. The presence of a stable metal-independent form of PP1 in the GSK-3 activated complex has been demonstrated after its removal from the activated complex (7).

3.4. The glycogen bound form of phosphorylase phosphatase - the targeting hypothesis

While studies of the purified phosphorylase phosphatase catalytic subunit showed that it had a broad specificity and could dephosphorylate both glycogen synthase and phosphorylase kinase (8,26,27), there remained a debate as to whether a separate synthase phosphatase and phosphorylase phosphatase existed in cells. Several observations fueled this debate, including partial separation of phosphorylase phosphatase and synthase phosphatase activities (28,29), as well as a dissociation of the physiological regulation of the two activities (30,31). Firstly, the dephosphorylation of liver phosphorylase and glycogen synthase were found to be sequential, and could be explained if phosphorylase *a* acted as a negative regulator of glycogen synthase phosphatase. Secondly, the ratio of synthase phosphatase/phosphorylase phosphatase activities of tissue extracts differed from that of the purified catalytic subunit, and thirdly, there was evidence that in fresh mouse tissue extracts, synthase phosphatase was largely associated with glycogen. In muscle, earlier studies had shown the existence of a "glycogen particle" in which glycogen sedimented from muscle extracts was shown to be associated with phosphorylase, phosphorylase phosphatase and phosphorylase kinase (32,33). The discrepancies were resolved by the purification of the glycogen-bound form of muscle phosphorylase phosphatase, which revealed it to be a heterodimer of the catalytic subunit and a glycogen binding subunit, G_M (8, 34). The glycogen binding subunit bound both glycogen and PP1, and also modified its substrate specificity in that its activity toward glycogen synthase was enhanced. These studies led Cohen (8, 35) to propose that the catalytic subunit is associated with different targeting subunits, which serve to direct the enzyme to specific subcellular localities, in addition to serving regulatory functions. This was the first evidence for a targeting function of PP1 binding proteins and was followed by isolation of the myosin bound PP1 which contains two other subunits, one of which, M_{110}, binds to myosin (36). The targeting hypothesis is substantiated by the recent discovery of a number of PP1 binding proteins, and the current view that the catalytic subunit exists in many heterodimeric forms provides an understanding of some of the original difficulties encountered in the isolation of the phosphorylase phosphatase enzyme.

4. RECOMBINANT PROTEIN PHOSPHATASE-1 CATALYTIC SUBUNIT

4.1. Molecular cloning and expression of the PP1 catalytic subunit

Ser/thr protein phosphatase genes have been cloned from a wide range of phyla (37) since the initial cloning of the cDNAs for rabbit muscle PP1α (38,39). Four isoforms of PP1 are known, these being PP1α, PP1γ1, PP1γ2 and PP1δ (40). PP1 is among the most conserved proteins known (41). The catalytic subunits of PP1, PP2A and PP2B share significant sequence identity (41), and thus are members of the same ser/thr protein phosphatase family, despite their nomenclature which was largely based on substrate specificity and inhibitor sensitivity (8).

Attempts to express PP1 as a recombinant p rotein were initially unsuccessful as it was readily overexpressed in the pET3a vector, but as an inactive, insoluble protein in inclusion bodies (42). Attempts to renature the solubilized PP1 protein were only partially successful, and required the presence of Mn^{2+}, dithiothreitol, high salt and high dilution. The renatured enzyme exhibited only 5% of the expected specific activity. The expression of PP1 in the pET3a vector as an insoluble material, with the attendant problems of renaturation and isolation made it an impractical system, so that we explored the use of other vector systems. We turned to the pTACTAC vector which had been used for the expression of rabbit muscle phosphorylase in a soluble form on the suggestion of Michelle Browner (42). This vector allowed the expression of PP1 in an active soluble form (43). Expression was dependent on induction at a lowered temperature of 26-28°C, rather than at 37°C, and was also dependent on the presence of Mn^{2+} in the culture media as had been used for the expression of phosphorylase (42). [When cultured in the absence of $Mn^{2+,}$ the purified enzyme exhibited a low specific activity (unpublished observations.)] The level of expression was approximately 4% of the soluble protein and recoveries of 5 mg purified PP1 protein per liter of culture were routinely obtained after a simple purification procedure. The recombinant PP1 could also be purified by a single chromatography step on inhibitor-2-Sepharose (44). PP1 has also been expressed in Sf9 cells; the enzyme is also insoluble but can be renatured (45). The use of the pTACTAC vector for the expression of PP1 has been reproduced in other laboratories (46-47).

4.2. Enzymatic properties of recombinant PP1

The striking feature of the recombinant protein which marks it as distinct from the catalytic subunit isolated from muscle and liver is that its activity is dependent on the presence of added Mn^{2+} (43). Muscle PP1 is normally isolated in the presence of buffers containing EDTA,

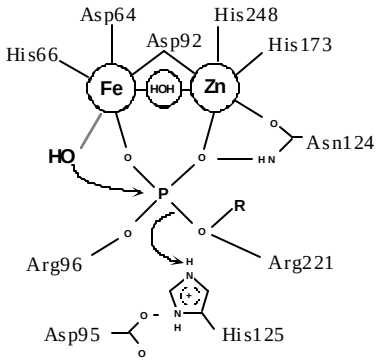

Figure 1. Active site of PP1. The active site of PP1 contains a bimetal center (shown as Fe and Mn) bridged by a water molecule which is displaced on binding of phosphate (55). Ligands for the metal ions are shown in heavy lines. The hydrogen bonds of the phosphate oxygens to N124, R96 and R221 are shown as thin lines. The catalytic mechanism, based on studies of PP1 (55), the purple acid phosphatase (61) and PP2B (57) is indicated in the diagram. The proposed reaction mechanism involves nucleophilic attack by a hydroxide ion, with H125 providing the proton for the leaving alcohol group, and is supported by mutagenesis of the active site residues (65).

and such preparations are also active in the presence of EDTA (10,18). The exceptions are the catalytic subunits released from the PP1-inhibitor-2 complex (10) or from high molecular weight forms of PP1 by trypsin (48). In addition, a form of cardiac myofibrillar PP1 that requires activation by Co^{2+} has been reported (49). We have suggested the recombinant enzyme may be related to the form present in the PP1-inhibitor-2 complex (50). The existence of the metal-dependent and -independent activity forms of PP1 suggests that there exist two stable conformations of PP1. The possible biochemical basis for these two conformations is discussed below (Section 3.1) in terms of the metalloprotein nature of PP1.

The specific activities and general enzymatic properties of recombinant PP1 toward phosphorylase *a* are similar to those of the muscle enzyme, with some exceptions. It is much less sensitive to inhibitor-1 (47,51), and dephosphorylates phosphotyrosine containing substrates (52). The latter findings are surprising and raise interesting questions regarding the structural basis of the difference of recombinant enzyme from the native enzyme which does not act on phosphotryosine substrates (see Section 3.1). Recombinant PP1 actively dephosphorylates p-nitrophenyl phosphate (43), a property that makes it useful for the assay of toxins (by inhibition of PP1 activity) such as microcystin by simple colorimetric methods (53,54).

5. STRUCTURE-FUNCTION STUDIES OF RECOMBINANT PP1

During the past 4 years there has been major progress in the elucidation of the atomic structures of the ser/thr protein phosphatases in general. The development of an expression system for PP1 has accelerated structure-function studies of PP1. Crystal structures for the PP1-microcystin (46), PP1-tungstate (55) and PP1-G_M peptide complexes (56) have been determined, in all cases of the recombinant PP1. Two structures for calcineurin (PP2B) have been solved, the auto-inhibited enzyme and a ternary complex of a truncated PP2B with a immunophilin-immunosuppressant drug complex (FKBP12/FKBP506) (57,58). These structures (for reviews see 59,60) show that the molecular architecture of the catalytic cores of PP1 and PP2B are conserved, and that both contain a bimetal center at the active site which is structurally similar (61) to that present in the purple acid phosphatase (PAP). The bimetal center is located in the active site and plays a crucial role in the binding of phosphate and as the source of the hydroxide ion that is the nucleophile in the catalytic mechanism (59,60).

5.1. The metalloprotein nature of PP1

The issue of whether PP1 is a metalloprotein had been an unresolved issue for many years, as there had been reports of PP1 activities which exhibited a dependence on added metal ions (reviewed in 10). Attempts to determine whether PP1 or PP2A contained metal ions provided equivocal results as only sub-stoichiometric levels of Fe and Zn were found (62). It was also shown that radioactive Mn^{2+} was not stably incorporated into PP1 under conditions where it stimulated PP1 activity (48). However, there were other lines of reasoning that suggested that PP1 might be a metalloprotein, including the conservation of primary structures of PP1 with the catalytic subunit of calcineurin (PP2B), which has stoichiometrically bound Fe and Zn (63). In collaboration with Dr K.K. Schlender, we demonstrated that $^{57}Co^{2+}$ can be stably incorporated into recombinant PP1 in a 1:1 stoichiometry (64).

The solution of the crystal structures of recombinant PP1 revealed that PP1, like calcineurin (57,58), is a metalloprotein that possesses a bimetal center that is bridged by a water molecule at the active site. The use of proton induced X-ray emission spectroscopy revealed the presence of Mn and Fe in the ratio 1 to 0.5 in PP1 (55). The nature of the metal ions in wild-type muscle PP1 is unknown, although it can be speculated that this may be an Fe/Zn pair as in PP2B (58,63). The coordination spheres of the metals in PP1 and PP2B are highly conserved with those of the purple acid phosphatases, whose oxide bridged bimetal center have been extensively studied by kinetic and biophysical methods (61).

Current views of the catalytic mechanism (reviewed in 60) for PP1 are that the metals serve as ligands for the phosphate oxygens, and for the generation of a hydroxide ion which serves as the nucleophile that is involved in catalysis, while H125 serves as a proton donor for the leaving alcohol group (figure 1). Other residues in the active site which serve to stabilize the proposed pentacoordinate state of the phosphate intermediate are R96, N124 and R221. We have performed site directed mutagenesis studies of the metal and phosphate binding ligands of PP1 (65), which have supported the catalytic mechanism inferred from the structure by Egloff *et al.*

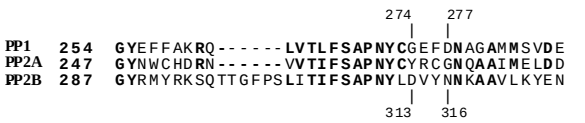

Figure 2. Alignment of C-terminal regions of PP1, PP2A and PP2B. The sequences of PP1, PP2A and PP2B in the region around C269 are shown. The boxed region shows the loop regions that connect beta sheets 12 and 13 in the structures of PP1 and PP2B (see figure 6). This loop is conserved except for the residues between 274-277 (GEFD) of PP1, 267-270 (YRCG) of PP2A, 312-316 (LDVYN) of PP2B, and the last residue in the box.

(55,60). Mutation of the metal ligands H66, D64 and D92 and H248 led to severe loss of catalytic function. Mutation of H125 and of H173 did not result in readily expressed proteins, although small amounts of the H125S and H125A mutants could be isolated. These mutants were inactive. Mutation of D95, which is proposed to stabilize the protonation of H125 by a salt bridge in PP1, resulted in significant reduction in catalytic activity. These findings support the idea that H125, stabilized by a salt bridge to D95, is essential for catalysis because it functions as the donor of a proton for the leaving alcohol group, as is proposed for PP2B (56). Mutation of R96, N124 have also supported their proposed roles in phosphate binding.

It has now become evident that the crystal structure of either native PP1, or a recombinant form that is functionally identical to native PP1, will be important to understanding the behavior of PP1. This emerges from the fact the recombinant enzyme differs from the muscle enzyme in having a reduced sensitivity to inhibitor-1 (51), and a striking ability to hydrolyze phosphotyrosine substrates (52), quite at odds with its identification as a ser/thr protein phosphatase. While the recombinant form of PP1 has provided the means for extensive structural studies, there may be concerns as to whether it represents a meaningful form of enzyme to study, because of its differences from the native enzyme. In addition, it has been suggested that that recombinant PP1 is a nascent form that requires the function of a chaperone in the form of inhibitor-2 (47,52). The demonstration that Co^{2+} can be stably incorporated into PP1 (64) suggests that these differences may be dependent on the metal occupancy of the two metal sites of PP1. For example, the Co^{2+} containing form is likely to be a Fe/Co metalloprotein, while the Mn^{2+} dependent form may be an hemi-apoprotein which contains only Fe, thus explaining its requirement for exogenous Mn^{2+}. Alternatively, the dependence on exogenous Mn^{2+} could reflect a situation that requires a substrate-directed binding of a third metal ion. In this regard, the fact that PP1 is a metalloprotein requires a recognition that variances of its properties may be a result of the existence of metallo-isoforms. Even native PP1, which is generally isolated in the presence of metal chelators, may have a compromised metal content. A recombinant form of PP1 containing Fe and Zn would likely answer many of these questions, but a method for generating this form has yet to be found.

5.2. Evidence for a common binding region for a number of structurally diverse toxins

A number of natural toxins of freshwater or marine origin are potent inhibitors of PP1 and PP2A (66). These include the polyether-like compounds, okadaic acid (67,68), calyculin A (69) and tautomycin (70). Okadaic acid, a fatty acid polyether, accumulates in certain sponges and shellfish and is the agent responsible diarrhetic poisoning (67). It has been widely used as a reagent for probing the cellular effects of the inhibition of phosphatase activity, and displays a greater potency toward PP2A than for PP1. Calyculin A, an octamethyl polyhydroxylated C28 fatty acid isolated from marine sponges, exhibits similar cytotoxic and tumor promoting activities as okadaic acid (67,71). Tautomycin, a polyketide toxin from a soil bacterium, has also been identified as a potent phosphatase inhibitor (14). A second group of toxins of freshwater origin are also potent inhibitors of PP1 and PP2A. These are the microcystins (72), a family of cyclic heptapeptide toxins, and a pentapeptide toxin, nodularin which inhibit PP1 and PP2A at nanomolar concentrations (73-75). These toxins which are produced by cyanobacteria constitute both acute and chronic hazards to human and animals (76).

Several indirect lines of evidence have shown that the binding of okadaic acid interferes with the binding of microcystin and tautomycin, suggesting that there may be a common binding site (70). More direct evidence that these toxins had a common contacts with PP1 was obtained by the generation of site-directed mutants of PP1, which exhibited a general loss of sensitivity toward several of these toxins. We obtained the evidence for a region of PP1 that was involved in toxin binding, based on the observations of Dr Minako Nagao that an okadaic acid resistant cell line contained a mutated PP2A (Cys269 to glycine) that had a reduced sensitivity to okadaic acid (77). In comparing the sequences of PP1 and PP2A in this region (figure 2) we noted that the region was well conserved except for a four residue difference, YRCG in PP2A (267-270) and GEFD (274-277) in PP1. The chimeric PP1 mutant in which GEFD was changed to YRCG resulted in an increased sensitivity to okadaic acid (78), consistent with fact that PP2A is more sensitive than PP1 to okadaic acid. This provided the impetus for further mutation of the region between residues 268-277 (79). Each of the mutants was characterized in terms of their sensitivities to okadaic acid, microcystin, nodularin, calyculin A and tautomycin (figure 3). These experiments also confirmed that the single mutation of F276 to cysteine caused an increase in the sensitivity of PP1 to okadaic acid (79). The differential sensitivity of PP1 and PP2A can be attributed largely to the presence of a cysteine residue at position 269 in PP2A and a phenylalanine residue at the cognate position (residue 276) in PP1.

The experiments also revealed that Y272 was important for the binding of all of the inhibitors tested, as its conservative mutation to phenylalanine caused large perturbations (decreases) in the toxin sensitivity of PP1 (figure 3). A systematic mutation of Y272 was also performed (79), and the results show that very large changes in sensitivity of PP1 to the polyether toxins,

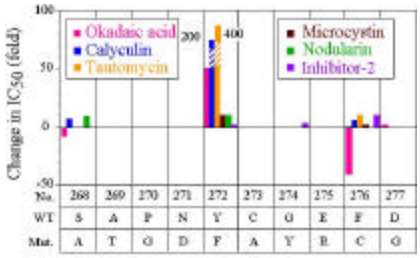

Figure 3. Effects of mutations at residues 268 to 277 on the toxin sensitivities of PP1. The single point mutants S268A, A269T, P270G, N271D, Y272F, C273A, G274Y, E275R, F276C, and D277G of PP1 were expressed and purified to homogeneity (79). The IC50's for okadaic acid, microcystin-LR, nodularin, tautomycin, calyculin A and inhibitor-2 were determined for each of these mutants (79). Data are expressed as the fold changes in IC50 (mutant/wild type for the positive values and negative values are the fold decreases in IC50 (wild type/mutant).

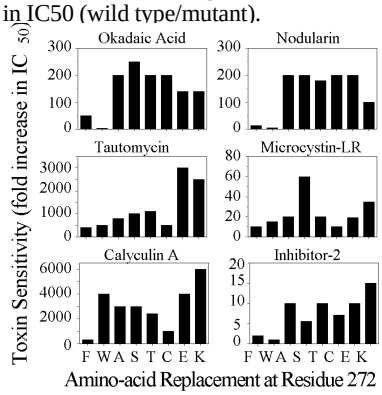

Figure 4. Effects of various amino acid replacements of Y272 on the toxin sensitivity of PP1.Mutations of Y272 to F, W, K, E, S, A, C and T were expressed and purified to homogeneity. The IC50s for okadaic acid, microcystin-LR, nodularin, tautomycin, calyculin A and inhibitor-2 were determined and compared to those of the wild type. Data are shown as fold changes in IC50 (79).

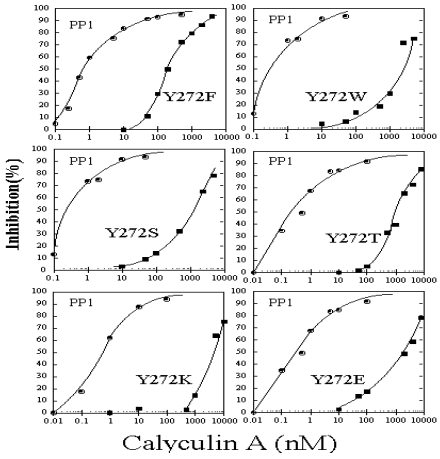

Figure 5. Inhibition curves for mutations of Y272 by calyculin A. The inhibition of the Y272 mutants are shown as a function of inhibitor concentration. Inhibitors tested were okadaic acid, tautomycin, calyculin A, microcystin-LR, nodularin, and inhibitor-2 Enzyme activity was assayed using phosphorylase *a* as the substrate (79).

particularly for calyculin A and tautomycin could be induced (figure 4).

This was particularly pronounced in the case of tautomycin and calyculin A, such that these mutants were unaffected by concentrations of toxin which could completely inhibit the wild type enzyme as shown for calyculin A (figure 5). These mutagenesis studies indicate that binding of the toxins must all involve some common contacts on PP1, and that Y272 is particularly important in this context.

Y272 is located close to the active site with its hydroxyl within several angstroms from the Fe ion (55); in the purple acid phosphatases the cognate tyrosine residue is co-ordinated with Fe, and is responsible for the color of the enzyme. The mutation of Y272, without deleterious effects on its catalytic activity, suggests that it is unlikely to be involved in catalysis in PP1.

The region of PP1 which we studied in fact represents the loop region connecting beta strands 12 and 13 in the PP1 structure. The structure of the PP1-microcystin complex (46) revealed that microcystin binds to the active site, with an glutamate residue binding to the metal ions, while its hydrophobic ADDA group is inserted along a hydrophobic groove in PP1 (46). The structure also identified C273 as the site of the covalent adduct formation with microcystin (46), consistent with other evidence by site directed mutagenesis (80). The microcystin-PP1 structure also provides important insights into the possible binding of substrates and the peptide inhibitors (see Section 3.4). Our mutagenesis studies of the loop region strongly suggests that the mode of inhibition of microcystin (active site occlusion) is common for the toxins that we tested. The location of the loop is shown in figure 6. If the toxins bind to the same site on PP1, this must reflect the possibility that these diverse molecules must present topographically similar surfaces at the points of interaction with PP1. This concept has received support from molecular modeling studies (81-83).

5.3. Studies of calcineurin (PP2B): Evidence for the importance of the b12/b13 loop in the regulation of PP2B

The loop connecting β strands 12 and 13 extends from the surface of PP1 and overhangs the active site. This loop forms a connection between the two beta sheets that provide the scaffold for the active site in both PP1 and PP2B (figure 6). We have proposed that the exposure of the loop and its connection to these two beta sheets provides a site for torsional effects on the two beta sheets that could provide a mechanism for the inhibition of PP1 catalysis, other than the occlusion of the active site. Further evidence for the significance of the β12/β13 loop the regulation of the ser/thr phosphatases comes from work on PP2B. Our interest in PP2B arose because we were drawn to examine the cognate region (figure 2) to that in PP1 (274-277) which we had found to be involved in toxin sensitivity. We speculated that the lack of sensitivity of PP2B to okadaic acid and microcystin might

Figure 6. The β12/β13 loop of PP1. The diagram shows a ribbon model of the PP1 structure (46) using the RasMol program. The two beta sheets that are the scaffold for the active site are shown in yellow (beta sheet 1) and magenta (beta sheet 2). Beta strands 12 and 13 are shown in red. Microcystin is shown in wireframe, and the two metal ions as black spheres.

have arisen from the difference in this region, and that this might be modified by generation of a chimeric PP2B mutant containing the PP1 or PP2A sequence. We developed an expression system for the A and B subunits of rat PP2B in *E. coli* which allows the preparation of mg amounts of pure A and B subunits (84). The recombinant A and B subunits could be reconstituted to a functionally active enzyme which was indistinguishable from that isolated from brain tissue. The PP2B A-subunit mutants that we studied (85) included two chimeric mutants inwhich a four amino acid stretch in the cognate L7 loops of the related protein phosphatase-1 (GEFD) or -2A (YRCG) were substituted for the calcineurin sequence DVYN (313-316), a point mutation (L312C), and a truncated mutant in which the YRG sequence replaced residues 313-316. These mutations did not affect the okadaic acid sensitivity of PP2B, but did lead to surprising results that turned out to be important in understanding the regulation of PP2B.

The regulation of PP2B is complex, in that it possesses an autoinhibitory C-terminal region, and is activated by Ca^{2+} which exerts its effects via calmodulin and a B-subunit which is structurally related to calmodulin. The crystal structure of the PP2B/FK506/FKBP12 complex (57) shows that the β12/13 loop is involved in an interaction with FKBP12, while the structure of the PP2B alone shows that the β12/13 loop is involved in the binding of the C-terminal autoinhibitory loop (58). The contact residues are revealed to be Y315 and N316, two of the residues which we had replaced.

The YRCG mutant was found to have a similar activity to the wild type enzyme, but its truncation mutant YRG was found to be hyperactivated (84), either as a free subunit or as the heterodimer. This effect could not be explained by loss of an interaction of the autoinhibitory C-terminus, since we performed additional studies in which this region was deleted. Thus, our experiments showed that the catalytic *core* of the A-subunit is in an inactive conformation. The effect of the shortening of the β12/13 loop in activating PP2B can be interpreted as one that results in a torsional stress on beta strands 12 and 13 that leads to activation of the A subunit (84). These studies provide evidence that the β12/13 loop is an important structural element in the conformation of the active site, and may participate in the conformational transitions of calcineurin between a catalytically repressed state and an activated state (84).

5.4. Substrate binding: Role of the acidic groove, active site and hydrophobic grooves

The active site of PP1 lies at the confluence of three shallow grooves, a C-terminal groove, an acidic groove and a hydrophobic groove, which are potential binding sites for substrates and inhibitors (46,55). Microcystin, a cyclic heptapeptide inhibitor, binds in a manner such that it occupies the active site, while its extended ADDA side chain occupies the hydrophobic groove. The hydrophobic groove forms the obvious binding site for peptide substrates. The two PP1 inhibitors, inhibitor-1 and DARPP-32 both carry four basic residues N-terminal to the phosphothreonine residue and its binding to PP1 has been hypothesized to be that of a pseudosubstrate (46). Binding of peptide/polypeptide substrates to PP1 can thus be considered to be composed of three elements: interaction of the basic residues N-terminal to the phosphoserine with the acidic residues in the acid groove, binding of the phosphoserine to the active site, and an interaction of the region C-terminal of the phosphoserine (or phosphothreonine) to the hydrophobic groove.

In the active site region, the structure of the PP1-tungstate complex has shown that R96, N124 and R221 are likely to be involved in the binding of the phosphate oxygens (55). R221 and R96 are well positioned to form salt bridges with two of the phosphate oxygens while the amino group of N124 can be hydrogen bonded to the third oxygen. D208 was hypothesized to be important for the orientation of R221 via a salt bridge interaction. Thus, these residues were proposed to be involved in the recognition of phosphate and also the proper orientation of the phosphate during the formation of the transition complex (55). Egloff *et al.* (55) have also pointed out that W206 and Y134 are well positioned to interact with the ser or thr carrying the phosphate residue. These interactions are shown diagrammatically in figure 7, and the location of the acid and hydrophobic grooves in figure 8.

We have systematically mutated residues in the acid groove, the active site region and the hydrophobic groove to assess the importance of these structural features in substrate recognition to test these hypotheses (86). The kinetic behavior of the mutant proteins toward phosphorylase *a*, RII peptide, Kemptide and *p*-nitrophenol phosphate as substrates were determined (86). Our studies showed that mutation of any one of the acidic residues in the groove did not markedly perturb binding of phosphorylase *a* , although effects were observed with the

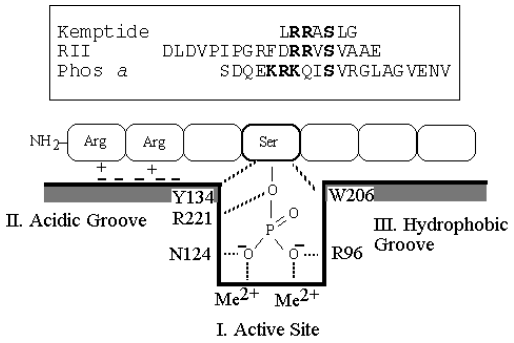

Figure 7. Binding of a peptide substrate to PP1. The binding of a peptide substrate to PP1 is shown diagrammatically, and is based on the crystal structures (46,55). **I**. Binding of the serine phosphate to the active site. Phosphate is orientated by interactions of the oxygens the two metals, with R96 and R221 by salt bridges, and by a hydrogen bond between N124 and a third oxygen. Serine could interact with Y134 and W206. II. Ionic interactions between the negative surface change of the acid groove with the basic residues of a peptide containing the PKA consensus sequence. III. A hydrophobic interaction with the C-terminal portion of the peptide with the hydrophobic groove of PP1. The sequences at the top show the sequences of the substrates used for analysis of mutations of residues in this region of PP1 (86).

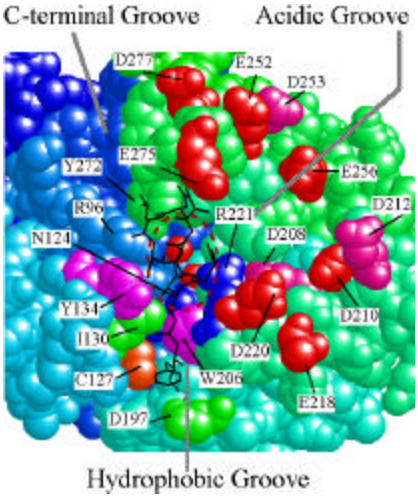

Figure 8. The C-terminal, acidic and hydrophobic grooves of PP1. The diagram shows a spacefilled model of PP1 with a view of the face of the molecule containing the active site. The dashed red oval shows the location of the active site, with the two metal ions as red spheres. Microcystin is shown in wireframe. The location of the hydrophobic groove is delineated by the binding of the ADDA group of microcystin. The hydrophobic, acidic and C-terminal grooves radiate from the active site. Residues which are labeled are those which were analyzed by mutation (86).

peptide substrates. Strong effects were only observed for mutation of residues involved in phosphate binding and orientation at the active site. Thus, the interaction of the N-terminal region of the phosphopeptide substrate is of lesser importance than the binding at the active site region. The kinetic behavior of mutants of R221, D208, and W206 support the proposal (55) that R221 plays an important role as a phosphate oxygen ligand which positions the substrate for catalysis. The kinetic behavior of mutants at W206 and D208 support the hypothesis that these residues (together with R221) form the microenvironment which dictates the orientation of the imidazole ring of H248, one of the metal binding ligands, as well as contributing to the orientation of R221 itself. In collaboration with Dr S. Shenolikar, Duke Univ., a number of these mutants have been analyzed for their sensitivity to inhibitor-1 (51,87).

6. Targeting of Protein Phosphatase-1- One Catalytic Subunit, Many Partners

Targeting, as the requirement for the molecular juxtaposition of proteins for the generation of signalling events, is well established as a paradigm in a number of growth regulated signalling systems involving tyrosine phosphorylation (88-91), as well as in the anchoring of ser/thr protein kinases by A-kinase anchoring proteins (AKAPs), one of which also binds PP2B (91,93). The concept of targeting as it relates to PP1, however, has a major twist to it in terms of the large number of PP1 binding proteins that have been reported during the past several years, as it expands the likely number of PP1 heterodimers that may exist, and consequently the repertoire of cellular functions that involve PP1.

6.1. PP1-binding proteins

Mammalian PP1-binding proteins which were either isolated by biochemical methods or identified by yeast two hybrid screens include the retinoblastoma gene product, pRb (94), HSP78 (95), p53BP2 (96), splicing factor PSF (97), ribosomal protein L5 (98), herpes virus $\gamma_1$34.5 protein (99), a human SDS22 homolog (100,101), NIPP-1 (102-104) RIPP-1 (105), HOX11 (106), spinophilin (107), p99 PP1 inhibitor (108), CP117 (109) and inhibitor-3 (110). In addition, several G_M related proteins (G_L, R5, U5, and PTG) have been identified (111-114). Genetic studies of yeast mutations that affect glycogen metabolism and cell cycle regulation, and the use of the two hybrid screens have revealed over a dozen genes that encode putative PP1-binding proteins (reviewed in 115-116). These include *GAC1*, *REG1*, *REG2*, *SCD5*, *GIP1*, *SHP1*, *GIP2* and *SDS22* in *S. cerevisiae* (115-116). These genes are variously required for control of glycogen metabolism, protein synthesis, glucose repression, meiosis and/or sporulation, and mitotic cell cycle regulation. Rigorous *biochemical* demonstration that these PP1-binding proteins actually interact with PP1, or the nature of the targeting functions or the substrates have not been shown in most of these cases.

A key element of the targeting hypothesis is that the cellular activity of PP1 is only expressed when it is targeted, *i.e.*, specificity is achieved by targeting of PP1 to a restricted microenvironment. This would rationalize the fact that PP1 is a relatively nonspecific phosphatase. The strongest experimental support for a targeting function for a PP1 subunit has come from genetic and biochemical studies of yeast glycogen metabolism. The glycogen-deficient yeast mutant *glc7-1* was found to have a PP1 with a R73C

Table 1. Alignment of peptide sequences that bind to PP1.

Group	Sequence	No. Occurrences
A 1	V R H K R **V R F** E A D V	1
A 2	V R H K R **V R F** E A D D	3
A 3	L K R R **V R F** E D G S P	1
A 4	V K R **V R F** Q E Q G A A	2
A 5	V K R **V R F** R E Q G A A	1
A 6	K R **V R F** K E G R V N S	1
A 7	R R **V R F** K N V P G Q H	1
A 8	K K **V R F** S N I V R Q R	1
A 9	A K R K **V R F** V G D R L	1
A 10	R A K K Q **V R F** A D L R	2
A 11	R V K K Q **V R F** A D L R	1
A 12	R A K K Q **V R F** S D L R	1
A 13	V K R Q **V R F** A D A K R	1
A 14	R A I K Q **V R F** A D L R	1
A 15	L R M H **V R F** N E A V S	1
A 16	R W V K T E A R H **V R F**	1
A 17	R R R R V K **F** H N P G H	1
B 1	R R **V H F** D N G E S G A	4
B 2	R A R H **V H F** R K Q T R	1
B 3	S K R **V H F** G R G R P R	1
C 1	K N M R H **V S F** A D E V	4
C 2	K N M R H **V S F** A D E D	2
C 3	G G G R K G K A **V T F** S	1
D 1	S T R H **V H W** D D R E A	10
D 2	R V S R H **V H W** A D L E	7
D 3	K K R R **V H W** T S G Y Q	1
D 4	R R **V H W** V D T V V A G	1
D 5	R K K **V H W** D T S V G N	1
D 6	R K P M **V H W** S D R A G	1
D 7	R K P R **V H W** S D R A G	1
D 8	R S K Q **V H W** G P Q K P	1
D 9	R K V P G R A R R **V H W**	1
D 10	R K V P G A A R R **V H W**	2
D 11	G V V R P K G K K **V H W**	1
D 12	P K **V H W** E D S A N R G	2
D 13	L Q **V H W** Q A G D V S A R	1
E 1	K K R **V R W** V D Q A A C	5
E 2	S M G K R R **V R W** A T G	1
F 1	R G K R V M **W** A D E G D	1
F 2	R R **V T W** S D G R Q R Q	2
F 3	K R **V S W** V D K T R A R	1
F 4	K K L H **V A W** D V R A R	1
G 1	K N M R H **V S** L A D E V	1
G 2	P N **V H** L E M H A Q S R	1
G 3	S T R **V H** V W D D R E A	1
G 4	R E K R T K **H W** R D S V	1

The table shows the alignment of 79 peptide sequences that were obtained by analysis of the pFliTrx plasmids obtained after six rounds of panning against immobilized PP1 (125). Basic residues which occur N-terminal to the VxF/W motif, and acidic residues in the first and second positions after the motif are underlined.

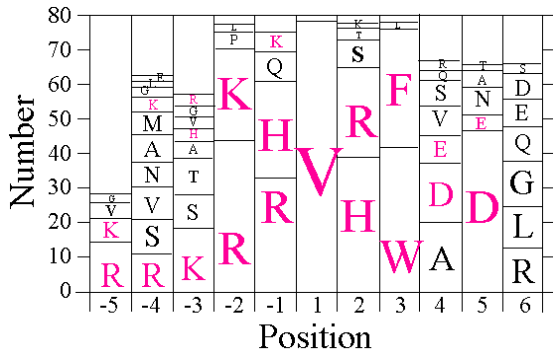

Figure 9. Frequency of amino-acids among the peptides in the PP1-binding motif. The diagram shows the frequency of amino-acid distribution at positions in the binding motif based on the data of table 1.

point mutation (123). This did not affect PP1 activity but resulted in a loss of its ability to bind to the yeast homolog (Gac1p) of the mammalian glycogen binding protein (124). The activation of glycogen synthase requires its dephosphorylation by PP1 activity, and it has been shown that glycogen synthase in this mutant is largely in the inactive phospho-form (124). Overexpression of Gac1p, on the other hand, led to increased glycogen accumulation (124). These findings demonstrated that the physiological functioning of PP1 in glycogen metabolism was totally dependent on its being targeted to the appropriate microenvironment, and importantly, other cellular functions of PP1 were not affected when targeting to glycogen was disrupted.

6.2. Molecular basis for multiple PP1 binding proteins

The growing number of PP1 binding proteins suggested that they possessed a peptide motif which was specifically recognized by a binding site on PP1. The existence of such a peptide motif that confers binding to PP1 was demonstrated the by panning of a random peptide display library (125). The random peptide library was one in which peptide sequences are displayed on the *E. coli* bacterial flagellin protein. The library was screened for bacteria which bound to PP1 immobilized on petri dishes. DNA sequence analysis of the encoded peptides from a total of 104 isolates was performed. Seventy-nine of these isolates harbored peptide sequences that were aligned into a common family and represent 46 unique sequences. These fell into two groups, one containing the motif VxF, where x is most frequently Arg, and one containing the motif VxW, where x is most commonly His. Both the VxF and VxW series were generally preceded by two to five basic residues immediately preceding the conserved valine. On the C-terminal side of the VxF/W motif, it was also evident that an acid residue was present in one of the next two residues. Thus, the consensus motif that emerges is R/K-R/K-X_{0-2}-**V**-R/H-**F/W**-X-D/E, where V or F/W are almost invariant. The frequency of amino-acids at various positions is shown in figure 9 and the peptide sequences in table 1..

Our results demonstrate that a short peptide sequence containing the general motif VxF or VxW preceded by one or more basic residues is sufficient to generate a capacity for the flagellin fusion proteins to bind to PP1. PP1 was structurally shown to possess a binding site for a peptide derived from the G_M subunit by the determination of the structure of its complex with PP1 (56). The sequence RRVSFA of G_M is bound to a hydrophobic channel, adjacent to a region of acidic residues that accommodates the N-terminal basic residue. This site is located on a side of PP1 that is nearly opposite to the active site, and is thus ideally situated as a site for the binding of targeting subunits.

An examination of the coding regions of a number of yeast genes encoding putative PP1-binding proteins and those of mammalian PP1 binding proteins (table 2) reveals the presence of this binding motif in the majority of cases (56,125). Interestingly, most of these contained the VSF sequence, which together with the N-terminal basic residues, form a PKA site. Thus,

Table 2. Sequences Corresponding to the VxF/W Motif In PP1-binding Proteins

Gene/protein	Residue	Sequence	Residue	Reference
S. cerevisiae genes				
GAC1	65	TSPEKN**VRF**AIE	76	X63941
GIP1	441	QKKKRC**V**N**F**RNK	452	Z35914
GIP1	486	DRSTSS**VRF**DEN	497	
GIP2	216	LIRSKS**VHF**DQA	227	U18813
GIP2h/YIL045W	191	LQRSKS**VHF**DRV	202	Z46861
GIP2h/YIL045W	411	KVFVKN I Y**F**DKK	422	
SCD5	26	GLGPPS**VSF**DFG	37	U03492
SCD5	268	NFKSKK**VRF**SEH	279	
YFL023W	31	DIRSRL**VRF**IND	42	D50617
YAL014	95	KESLKK**VRF**KND	106	L05146
YFR003c	35	MPTRHN**VRW**EEN	46	D50617
SHP1	260	ALKLLD**V**Q**F**GQE	271	Z35819
SHP1	344	CNSTDT**V**K**F**LYE	355	
Mammalian				
Rabbit G_M	60	SSGGRR**VSF**ADN	71	M65109
Human G_M	57	SSGTRR**VSF**ADS	68	X78578
PTG	56	NQAKKR**V**V**F**ADS	67	U89924
Human G_M	144	IIRVLN**V**S**F**EKL	155	X78578
PTG	149	TVKVKN**VSF**EKK	160	U89924
G_L	56	KKVKKR**VSF**ADN	67	S80360
M_{110}	30	KRQKTK**V**K**F**DDG	41	S74907
NIPP	195	KRKNSR**VTF**SED	206	Z50748
p53bp2	793	IAHGMR**V**K**F**NPL	804	U58334
p53bp2	846	AGHTEI**V**K**F**LVQF	858	
Splicing factor PSF	358	RGRQLR**VRF**ATH	369	X70944
Rib. protein L5	12	YFKRYQ**V**K**F**RRR	23	U14966
HSV $\gamma_1$34.5 protein	176	PATPAR**VRF**SPH	187	M33701
Hamster GADD34	500	PLRARK**VHF**SEN	511	L28147
DARPP32	3	PKDRKK IQ**F**SVP	14	M27444
Inhibitor-1	4	DNSPRK IQ**F**TVP	15	J05592

The table lists sequences in PP1-binding proteins which contain the VxF/W motif (shown in bold letters). Basic residues preceding the VxF/W motif and acidic residues following the motif, are underlined. References are to the Genbank accession numbers. G_M, G_L, and PTG are glycogen binding proteins; the yeast genes *GAC1, GIP1, GIP2, GIP2h* are yeast homologs of G_M. *SCD5* is essential for cell growth and is involved in vesicular transport. GADD34 is the hamster homolog of HSV $\gamma_1$34.5.

phosphorylation at this site by PKA may provide a mechanism for disruption of the interaction with PP1, as has been shown for NIPP-1 and G_M (56,102). Both inhibitor-1 and DARPP-32 contain sequences at their N-termini (KIQF) that are similar to the RVxF motif, and are required for inhibition of PP1 (51,126).

Egloff *et al.* (56) have pointed out that the PP1 binding motif is present in perhaps as many as 10% of the proteins in the Genbank database. This raises an important caveat regarding the identification of PP1 binding proteins by the yeast two hybrid system, since it involves the interaction of fusion proteins which contain truncated versions of the test proteins. These interactions may not be evident in the native full length proteins, so that further biochemical analysis of these interactions is required for confirmation. Furthermore, positive identification of the motif sequences in PP1 binding proteins will require site directed mutagenesis studies.

It should also be noted that not all PP1 binding proteins necessarily harbor the peptide motif. Using an affinity chromatography approach with immobilized PP1 (127), we have found that phosphofructokinase binds to PP1 (128), but does not contain the motif.

7. PERSPECTIVE

The number of cellular functions in which PP1 has been demonstrated is quite extensive although rigorous biochemical definitions of these roles has not been achieved in the majority of cases. The original discoveries that mutants of *S. pombe* (129) and *A. nidulans* (130) that were blocked in mitosis carried mutations in PP1 genes revealed an essential function of PP1 in mitosis. Subsequent studies of the genetics of the PP1 gene yeast have now revealed a complex involvement of PP1 activity in cellular functions through multiple protein partners. It is noteworthy that the rabbit muscle cDNA which we cloned (38) has been shown to rescue the *A. nidulans* BimG11 mutant (131), a powerful argument that the understanding of PP1 functions in yeast can provide insights into its mammalian functions. In mammalian systems, there was more limited evidence for a role of PP1 in cell cycle control in the form of the dephosphorylation of Rb, whose phosphorylation is a key signal for the initiation of S (132). Recent work has now provided additional support for a role of PP1 in cell cycle regulation. Phosphorylation of Thr320 by cdc2 kinase has been shown to downregulate PP1 (133), and PP1 is highly phosphorylated only during early to mid-mitosis (134-

135). Expression of a PP1 mutant that lacked an inhibitory phosphorylation site for cyclin kinases at T320 was shown to result in an Rb-dependent cell cycle arrest at the G1/S border (136). A current view is that PP1 maintains Rb in the dephospho state during G1, so that termination of PP1 activity may be required for the shift of Rb to the phosphorylated state by cyclin dependent kinases (136). Inhibitor-2 has been suggested to have a role in this process, since its expression oscillates during the cell cycle (137), and inhibitor-2-fusion proteins are translocated to the nucleus at S-phase, consistent with a scenario in which PP1 activity must be shut down for the entry to S (138). Microinjection of PP1 in mammalian cells also supports a role for PP1 in mitosis (139).

The study of PP1 has passed a major threshold in the past 4 years in terms of our knowledge of its properties, its potential diversity of functions and its importance to control of cell proliferation. The concept that this single catalytic subunit has multiple targeting subunits via the existence of the PP1-binding peptide motif has important implications for our understanding of PP1 functions. From this perspective, PP1 may serve a large number of cellular functions, dictated by the individual targeting subunits. There now appears a new horizon for the study of this "old" phosphatase that lies in the identification and characterization of its targeting/regulatory subunits, and the *parallel* elucidation of their biochemical properties. Determination of the cellular functions of PP1 will be highly challenging, as they will likely be dictated by the properties and localization of the individual targeting subunits, and may be extremely diverse. Furthermore, the conserved nature of PP1 provides a clue to the evolutionary development of PP1 functions, *i.e.*, the concept that PP1, one of the most highly conserved proteins known, gained a large divergence of function through the acquisition of multiple targeting proteins (125).

The diverse involvement of PP1 in important cell functions make it almost inevitable that it will be implicated in pathogenic processes that have implications for health care in the future. Genetic alterations that affect PP1 or its regulatory proteins could obviously lead to changes that impinge on the growth characteristics of cells and thus be involved in the process of oncogenesis. In particular, PP1 regulatory subunits have a potential to emerge as potential tumor suppressors. The HOX11 gene which interacts with both PP1 and PP2A, has been shown to be oncogenic in human T-cell leukemia (106). In the case of the related enzyme, PP2A, a leukemia associated protein, SET, has been shown to be a potent inhibitor of PP2A (140). HRX fusion proteins which arise by translocations of the HRX gene in acute leukemia, have been shown to interact directly with SET (141). More recently, a direct search for a tumor suppressor gene has led to the identification of a PP2A regulatory subunit and the demonstration that it is altered in human lung and colon cancer cells (142). Intervention in protein phosphatase regulatory systems may also underlie mechanisms for viral infectivity. The acquisition of a gene encoding a PP1 targeting protein has been demonstrated as a mechanism for a viral strategy for overcoming host cell defenses in the case of the herpes virus protein $\gamma_1$34.5 (99). An understanding of the molecular basis for the interaction of PP1 with its targeting proteins could also provide the basis for future drug design.

8. ACKNOWLEDGMENTS

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