Protein phosphatase 1 regulation by inhibitors and targeting subunits

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Regulation of protein phosphatase 1 (PP1) by protein inhibitors and targeting subunits has been previously studied through the use of recombinant protein expressed in *Escherichia coli*. This preparation is limited by several key differences in its properties compared with native PP1. In the present study, we have analyzed recombinant PP1 expressed in Sf9 insect cells using baculovirus. Sf9 PP1 exhibited properties identical to those of native PP1, with respect to regulation by metals, inhibitor proteins, and targeting subunits, and failure to dephosphorylate a phosphotyrosine-containing substrate or phospho-DARPP-32 (Dopamine and cAMP-regulated phosphoprotein, M_r 32,000). Mutations at Y272 in the β12/β13 loop resulted in a loss of activity and reduced the sensitivity to thiophospho-DARPP-32 and inhibitor-2. Mutations of Y272 also increased the relative activity toward a phosphotyrosine-containing substrate or phospho-DARPP-32. Mutation of acidic groove residues caused no change in sensitivity to thiophospho-DARPP-32 or inhibitor-2, but one mutant (E252A:D253A:E256R) exhibited an increased K_m for phosphorylase *a*. Several PP1/PP2A chimeras were prepared in which C-terminal sequences of PP2A were substituted into PP1. Replacement of residues 274–330 of PP1 with the corresponding region of PP2A resulted in a large loss of sensitivity to thiophospho-DARPP-32 and inhibitor-2, and also resulted in a loss of interaction with the targeting subunits, spinophilin and PP1 nuclear targeting subunit (PNUTS). More limited alterations in residues in β12, β13, and β14 strands highlighted a key role for M290 and C291 in the interaction of PP1 with thiophospho-DARPP-32, but not inhibitor-2.

Protein phosphatase 1 (PP1) is a major eukaryotic serine/threonine protein phosphatase that regulates such diverse cellular processes as cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription, and neuronal signaling (1–4). The catalytic subunit of PP1 is regulated by the heat-stable protein inhibitor-1, its homologue DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, M_r 32,000), and inhibitor-2 (1, 5). Phosphorylation of inhibitor-1 at T35 or of DARPP-32 at T34 by cAMP-dependent protein kinase is required for PP1 inhibition. In contrast, unphosphorylated inhibitor-2 interacts with the PP1 catalytic subunit, leading first to the inhibition of enzymatic activity and, subsequently, to the formation of an inactive complex termed Mg-ATP-dependent phosphatase (6). PP1 also interacts with a variety of regulatory subunits that target the catalytic subunit to specific subcellular compartments (4). These include the glycogen-targeting subunits, G_M and G_L (7), the myofibrillar-targeting subunit, M_{110} (7), the nuclear-targeting proteins, nuclear inhibitor of PP1 (NIPP-1) (8) and PP1 nuclear targeting subunit (PNUTS) (9, 10), and the neuronal dendritic spine-targeting proteins, spinophilin and neurabin (11, 12).

Numerous studies have shown that PP1 interacts with DARPP-32/inhibitor-1 and targeting subunits via a common docking motif that consists of one or more basic amino acids followed by two hydrophobic residues that are separated by a variable amino acid (13–17). X-ray crystallographic analysis of G_M/PP1 has indicated that the docking motif (RRVSF) interacts in an extended manner with a hydrophobic channel in PP1 situated on the side opposite that of the active site (14). Residues that form the hydrophobic channel come from several β-strands, including β12, β13, and β14. In addition to the interaction via the docking motif, DARPP-32/inhibitor-1 (and possibly some of the targeting proteins) likely make additional contacts with β12, β13, and β14 such that the phospho-T34/T35 comes close to, or even occupies, the PP1 active site (5). In contrast, inhibitor-2 does not seem to contain a canonical RRVSF docking motif. Rather, inhibitor-2 possesses a unique N-terminal motif that interacts with a region of PP1 that is slightly removed from the hydrophobic channel discussed above (5, 18).

Several studies indicate that the β12, β13, and β14 strands play a key role in the highly specific interactions of protein inhibitors and targeting subunits with PP1 (14, 19–21). Indeed, replacement of the C terminus of PP1, including the β12–β14 strands, with the corresponding region of the structurally related protein phosphatase, PP2A, renders the chimeric phosphatase activity insensitive to inhibitor-1 and inhibitor-2 (22). Surprisingly, the PP1/PP2A chimera still apparently bound these proteins. To further analyze PP1 regulation by inhibitors and targeting subunits, many studies have used site-directed mutagenesis of the PP1 catalytic subunit expressed in *Escherichia coli* (20, 21, 23). However, *E. coli* PP1 differs from native PP1 in several important properties: it depends on added Mn^{2+} for activity; it is able to dephosphorylate phospho-tyrosine-containing substrates and *para*-nitrophenyl phosphate; it is relatively insensitive to inhibition by phospho-DARPP-32 and phospho-inhibitor-1, partly through the dephosphorylation of the phospho-inhibitors (16, 20, 24, 25); and it is insensitive to regulation by several targeting subunits (unpublished results).

In the present study, we have used a baculovirus expression system in Sf9 insect cells to produce a recombinant PP1 preparation that exhibited properties very similar to those of native PP1. We have used this enzyme preparation to carry out detailed structure–function studies of PP1. The results obtained have provided insights into the interaction of PP1 with inhibitor proteins, targeting subunits, and substrates.

Materials and Methods

Materials. Oligonucleotides were synthesized by Operon Technologies (Alameda, CA). The QuickChange site-directed mu-

Abbreviations: PP1, protein phosphatase 1; DARPP-32, dopamine and cAMP-regulated phosphoprotein, M_r 32,000; GST, glutathione-*S*-transferase; Ni-NTA, ni-nitrilotriacetic acid; PNUTS, PP1 nuclear targeting subunit; TEV, tobacco etch virus.

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tagenesis kit was from Stratagene. The Bac-to-Bac Baculovirus Expression System, including the vector plasmid pFastBac-HT, DH10BAC competent cells, and recombinant tobacco etch virus (TEV) protease, were from Life Technologies (Rockville, MD). Sf9 cells were from Novagen. Complete protease inhibitor mixture tablets and protease inhibitor E-64 were from Roche Molecular Biochemicals. NHS-Hi Trap and glutathione Sepharose were from Pharmacia Biotech (Uppsala, Sweden). Ni-nitrilotriacetic acid (Ni-NTA) agarose was from Qiagen (Chatsworth, CA). Immobilon-P was from Millipore; PNUTS peptide was synthesized and purified by reversed-phase column HPLC at W. M. Keck Foundation Biotechnology Resource Laboratory, Yale University. The Protein Tyrosine Phosphatase Assay System was from New England Biolabs. Monoclonal antibody for PP1 (E-9) was from Santa Cruz Biotechnology. The catalytic subunit of native PP1 was purified from rabbit skeletal muscle, and recombinant PP1 was purified from *E. coli* as described (20, 26). DARPP-32 and inhibitor-2 were prepared from *E. coli* as described (5). GST-tagged spinophilin and GST-PNUTS were prepared from *E. coli* as described (9, 27).

Preparation of Baculovirus. Rabbit PP1α cDNA was a generous gift from N. Berndt (Children's Hospital of Los Angeles, University of Southern California School of Medicine, Los Angeles, CA). *Eco*RI and *Xba*I restriction sites were added in the 5′end and 3′end of the PP1 sequence, and the cDNA was used as a template for site-directed mutagenesis using PCR. PP1/PP2A chimera cDNA was amplified by PCR using the CRHM2 plasmid that encodes residues 1–273 of human PP1α fused in frame with residues 267–309 of bovine PP2A (22). PCR products were inserted in frame into pFastBac-HT. For some constructs, cDNA encoding glutathione *S*-transferase (GST) followed by a TEV protease cleavage site, and a FLAG tag was introduced in frame between the 6xHis tag and PP1 sequences. Thus, proteins had a 6xHis tag, a TEV protease cleavage site, a GST tag, a second TEV cleavage site, and a FLAG tag at the N terminus of PP1. The QuickChange kit was used for site-directed mutagenesis.

Recombinant baculovirus stocks were prepared as described by the manufacturer (Life Technologies). In brief, donor plasmids were used for transformation of competent DH10BAC *E. coli* cells. Recombinant bacmid DNA was isolated and used for transfection of Sf9 cells. After 48 h incubation at 28°C, supernatant from the culture medium was saved and used for further amplification.

Expression and Purification of Wild-Type and Mutant PP1. Sf9 cells were grown in suspension culture (28°C, rotating at 135 rpm) in Grace's insect medium [supplemented with 0.33% yeastolate/0.33% lactalbumin hydrolysate/10% (vol/vol) FCS]. Sf9 cells (1.5×10^6 cells per milliliter) were infected at a multiplicity of infection between 5–10 plaque-forming units.

For purification of 6xHis-tagged PP1, cells ($\approx 2 \times 10^7$ per milliliter) were lysed with buffer containing 0.1 M KCl and 1% Nonidet P-40. Supernatants (centrifugation at 100,000 $\times$ *g* for 30 min) were incubated with Ni-NTA agarose and transferred into a column. The resin was washed with buffer containing 0.5 M KCl, 10% (vol/vol) glycerol, and 20 mM imidazole, and eluted with buffer containing 0.1 M KCl, 10% (vol/vol) glycerol, and 150 mM imidazole. Fractions containing active PP1 were dialyzed against buffer containing 50 mM Tris·HCl (pH 7.0), 50% glycerol, 0.1 mM EGTA, and 0.1% 2-mercaptoethanol. For purification of GST-tagged PP1, cells ($\approx 3 \times 10^7$ cells per milliliter) were lysed with buffer (0.1 M NaCl/1% Nonidet P-40). The supernatant (centrifugation at 100,000 $\times$ *g* for 30 min) was incubated with glutathione-Sepharose, and the resin was pelleted by centrifugation and washed with PBS. The resin was then resuspended in 50 mM Tris·HCl (pH 8.0)/10 mM EDTA/1 mM DTT/10% (vol/vol) glycerol, and incubated with 200 units of 6xHis-tagged TEV protease. After cleavage, Ni-NTA agarose was added to the suspension to remove the His-tagged TEV protease. The suspension was centrifuged, and the supernatant was used for protein phosphatase assay. For the various PP1 preparations, expression reached a maximum 48–72 h after infection. Approximately 50% of expressed PP1 was recovered in the soluble fraction, and $\approx$10 μg of purified protein was recovered from 1×10^8 cells in 100 ml of culture.

Immunoblotting of Recombinant PP1. Proteins were separated by SDS/PAGE and electrophoretically transferred to Immobilon-P membrane by using standard procedures. Membranes were incubated in PBS containing 0.1% Tween 20, 0.5% nonfat dry milk, and 1 μg/ml anti-PP1 monoclonal antibody (E-9), which also recognizes the PP1/PP2A chimera and all PP1 mutants. Rabbit anti-mouse IgG antibody and ^{125}I-labeled protein A were used for detection of signal. Radioactivity was quantified by using a PhosphorImager (Molecular Dynamics), and purified recombinant PP1 of known concentration was used as standard.

Protein Phosphatase Assays. Serine/threonine phosphatase activity was assayed by using as substrate [^{32}P]phosphorylase *a* or [^{32}P]DARPP-32 phosphorylated by cAMP-dependent protein kinase (26). Assays contained 0.01% (wt/vol) Brij 35, 0.3 mg/ml BSA, 10 μM [^{32}P]phosphorylase *a* or 1–5 μM [^{32}P]DARPP-32, various protein inhibitors, and PP1. Assays of *E. coli* PP1 contained 1 mM $MnCl_2$. For the assay of inhibitors, PP1 and inhibitors were preincubated for 15 min on ice. For peptide competition studies, PNUTS (392–408) and protein inhibitors were mixed before addition of PP1. Phosphorylation and thiophosphorylation of DARPP-32 were carried out as described (20). Tyrosine phosphatase activity was assayed by using as substrate [^{32}P]myelin basic protein phosphorylated by Abl protein tyrosine kinase prepared as described by the manufacturer of the Protein Tyrosine Phosphatase Assay System. All assays were performed in duplicate, and experiments were repeated at least two times.

Coprecipitation Assays. GST-tagged PNUTS (residues 309–691) or GST-tagged spinophilin (residues 298–817) (50 μg) was incubated with 50 μl of glutathione-Sepharose in 1 ml of binding buffer (20 mM triethanolamine (pH 7.0)/50 mM NaCl/10% (vol/vol) glycerol/0.1% 2-mercaptoethanol/1 mg/ml BSA) at 4°C with rotation. The resin was centrifuged and washed three times with binding buffer. Protein-bearing resin (20 μl) was incubated with $\approx$0.2 μM PP1 or PP1/PP2A chimera in 100 μl of binding buffer containing 0.5 mg/ml BSA at 4°C for 2 h. After three washes with 1 ml of binding buffer, protein coprecipitated with the beads was eluted by boiling in Laemmli sample buffer and analyzed by immunoblotting by using anti-PP1 antibody.

Results

Characterization of PP1 Expressed in Sf9 Cells. As discussed above, *E. coli* PP1 differs from native PP1 in several ways, limiting the interpretation of studies carried out with this preparation. Therefore, we assessed the properties of PP1 expressed in insect cells using the baculovirus method with that of native rabbit muscle PP1 and *E. coli* PP1. Sf9 PP1 exhibited properties essentially identical to those of native PP1, with respect to its lack of dependence on added Mn^{2+} (data not shown), its sensitivity to phospho-DARPP-32 and inhibitor-2 (Fig. 1 and Table 1), its failure to dephosphorylate tyrosine-phosphorylated myelin basic protein and phospho-DARPP-32 (Table 2), its ability to bind tightly to spinophilin and PNUTS, and its ability to be inhibited by these two targeting subunits (Fig. 2). Thus, Sf9 PP1 is a more suitable preparation for detailed structure–function analysis.

The C-terminal subdomain of PP1 (that includes β12–β14) sits on the surface of the N-terminal subdomain, forming three

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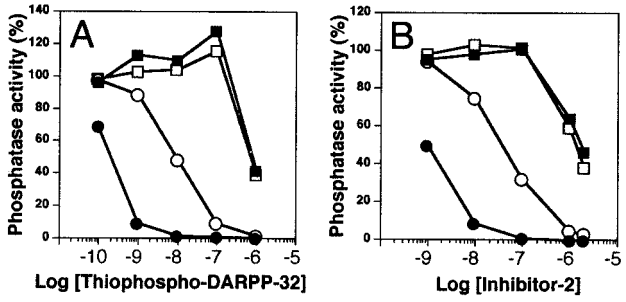

Fig. 1. Inhibition of wild-type Sf9 cell PP1 and PP1/PP2A chimera by thiophospho-DARPP-32 and inhibitor-2. Phosphatase activity of wild-type PP1 (closed and open circles) and PP1/PP2A chimera (closed and open squares) was measured in the absence (closed symbols) or presence (open symbols) of 20 μM PNUTS[392–408] peptide and the indicated concentrations of thiophospho-DARPP-32 (*A*), or inhibitor-2 (*B*). Phosphatase activity was measured by using 10 μM [^{32}P]phosphorylase *a* as substrate. Phosphatase activity is expressed as percent of activity measured in the absence of protein inhibitors.

surface grooves—the hydrophobic groove, the acidic groove, and the C-terminal groove—with the active site situated at the bifurcation point of the three grooves (28, 29). Several residues in the acidic groove (D220, E252, E256, and E275) are found only in PP1 (and not in PP2A or PP2B). PP1 preparations in which these residues were mutated were analyzed with respect to various enzymatic properties (Tables 1 and 2). Among the acidic groove mutants, only PP1[E252A:D253A:E256R] exhibited a significantly higher apparent K_m value than wild-type Sf9 PP1 or native PP1. Mutation of D220, E252, E253, E256, or E275, either singly or in combination, did not result in any significant change of sensitivity to thiophospho-DARPP-32 (Table 1).

A loop between β12 and β13 overhangs the active site and positions a highly conserved residue, Y272, close to the active site, but its function has remained unclear. Previous studies in which Y272 was mutated in *E. coli* PP1 indicated that this residue plays an important role in the interaction of PP1 with toxins like okadaic acid, microcystin, and calyculin A, as well as phospho-DARPP-32, phospho-inhibitor-1, and inhibitor-2 (19–21). Surprisingly, in these previous studies, mutation of Y272 had no effect on enzyme activity, despite the fact that this residue is conserved through evolution not only in PP1 but also in PP2A and PP2B. In contrast, Sf9 PP1[Y272F] exhibited a significant loss of activity, and Sf9 PP1[Y272A] exhibited a very large loss of activity, although some activity could be measured at high substrate concentration (Table 1 and data not shown). However, consistent with previous results, mutations of Y272 resulted in a large decrease in sensitivity to thiophospho-DARPP-32 and inhibitor-2, with a greater effect being observed for PP1[Y272A] than PP1[Y272F]. The effect of both mutations was greater for PP1 inhibition by thiophospho-DARPP-32 than that for inhibitor-2.

Table 1. Characterization of wild-type PP1 and PP1 mutants expressed in Sf9 cells

	K_m	V_{max}	S-D32 IC_{50}	I-2 IC_{50}
Wild-type PP1	3.7	18.5	0.7	1.5
D220V	1.6	7.3	0.8	ND
E256R	5.7	6.6	0.8	ND
E275R	3.5	3.9	3.0	ND
E252A:D253A	2.0	3.9	1.8	ND
E252A:D253A:E256R	19.5	2.1	ND	ND
Y272F	4.4	0.8	58	4.9
Y272A	ND	ND	179	54
PP1[GEFD > YRCG]	5.6	0.2	2.3	155
PP1[MC > KY]	1.6	6.4	58	78
PP1[QILK > LQFD]	6.1	1.9	0.7	23
PP1/PP2A Ch	1.8	1.0	400	1,583
PP1/PP2A Ch[YRCG > GEFD]	6.2	4.3	857	345
PP1/PP2A Ch[KY > MC]	1.4	1.8	5.9	1,000
PP1/PP2A Ch[LOFD > QILK]	1.1	0.7	675	2,050

[^{32}P]phosphorylase *a* was used as substrate. Apparent K_m (μM) and V_{max} (μmol/mg/min) are given. The IC_{50} values for thiophospho-DARPP-32 (S-D32) and inhibitor-2 (I-2) are in nM. ND, not determined. Both thiophospho-DARPP-32 and inhibitor-2 inhibit native rabbit muscle PP1 with IC_{50} values of ≈1 nM (5). Results are the average of two to six separate experiments.

Table 2. Relative activity of PP1 preparations towards tyrosine-phosphorylated myelin basic protein and phospho-DARPP-32

Strain	Phospho-tyr Myelin basic protein	Phospho-D32
Wild-type PP1 (*E. coli*)	100	100
Native PP1 (rabbit muscle)	0.5	0.5
Wild-type PP1 (Sf9)	2.4	0.8
PP1-Y272F (Sf9)	15.8	6.7
PP1-Y272A (Sf9)	900	150
PP1/PP2A Ch (Sf9)	1.8	2.3

Wild-type PP1 or PP1 mutants were expressed in *E. coli* or Sf9 cells as indicated. Native PP1 was purified from rabbit muscle. Phosphatase activity determined for either substrate was normalized to that determined using phosphorylase *a* as standard substrate. The relative phosphatase activities were then expressed as a percentage of that of wild-type PP1 expressed in *E. coli*. Results are the average of three separate experiments.

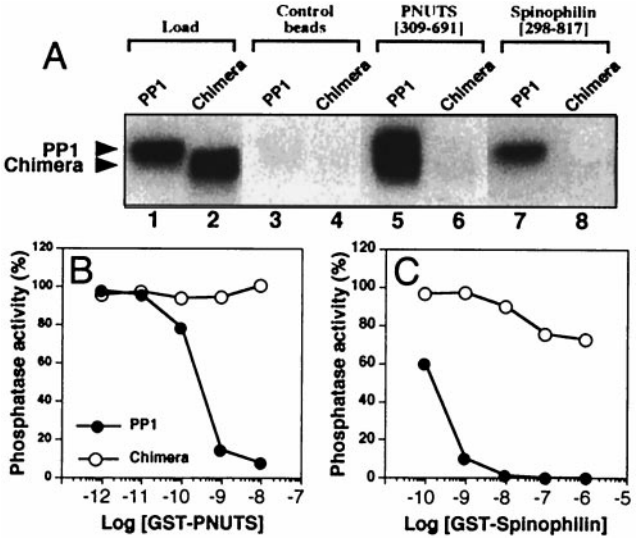

Fig. 2. Regulation of Sf9 cell PP1 and PP1/PP2A chimera by targeting subunits. (*A*) Coprecipitation of PP1 and PP1/PP2A chimera with GST-PNUTS and GST-spinophilin. Wild-type PP1 (lanes 1, 3, 5, and 7) or PP1/PP2A chimera (lanes 2, 4, 6, and 8) was incubated with control beads or beads bearing GST-PNUTS[309–961] (lanes 5 and 6), or GST-spinophilin[298–817] (lanes 7 and 8). After washing, bound PP1 or PP1/PP2A chimera was detected by immunoblotting. The loaded wild-type PP1 and PP1/PP2A chimera are shown in lanes 1 and 2 and indicated by arrowheads. The molecular weight of PP1/PP2A is less than that of PP1 because of its shorter C-terminal tail. Nonspecific binding to the bead matrix was negligible (lanes 3 and 4). (*B* and *C*) Phosphatase activity of wild-type PP1 (closed circles) and PP1/PP2A chimera (open circles) was measured in the presence of the indicated concentrations of GST-PNUTS[309–961] (*B*) or GST-spinophilin[298–817] (*C*). Phosphatase activity was measured as described in the Fig. 1 legend.

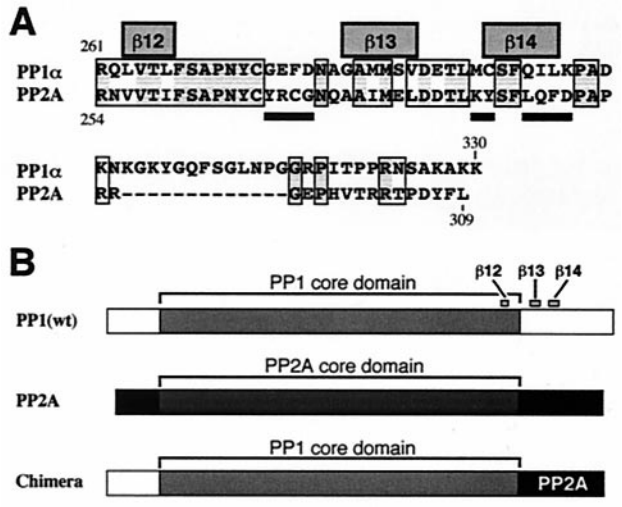

Fig. 3. Comparison of C-terminal domains of PP1 and PP2A and construction of PP1/PP2A chimeras. (*A*) Alignment of amino acid sequences of human PP1α and bovine PP2A. Conserved amino acids (including homologous substitutions) are boxed, and identical amino acids are indicated with a gray background. Positions of β-strands of PP1 are indicated by gray boxes above the alignment. Amino acids altered in the PP1/2A chimeras are underlined in bold. (*B*) Construction of PP1/PP2A chimeras. The highly conserved core region of PP1 (residues 41–273) and the corresponding region of PP2A are indicated by light gray and dark gray bars, respectively. N- and C-terminal variable regions of PP1 and PP2A are indicated by white and black bars.

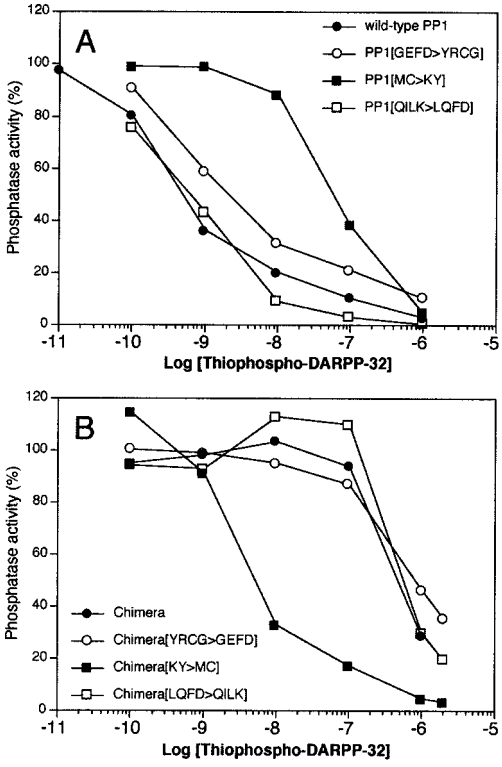

Fig. 4. Comparison of the regulation of PP1 and PP1/PP2A chimeras by thiophospho-DARPP-32. Phosphatase activity was measured in the presence of the indicated concentrations of thiophospho-DARPP-32. (*A*) Inhibition of wild-type PP1 and C-terminal domain mutants derived from PP1 as indicated. (*B*) Inhibition of PP1/PP2A chimera and mutants derived from the chimera as indicated. Phosphatase activity was measured as described in the Fig. 1 legend.

A distinct property of *E. coli* PP1 is its ability to dephosphorylate phospho-DARPP-32 and tyrosine-phosphorylated substrates (20, 25). Notably, Sf9 PP1, like native PP1, did not dephosphorylate phospho-DARPP-32 or tyrosine-phosphorylated myelin basic protein at any substantial rate (Table 2). However, mutation of Y272 resulted in an increase in the relative phosphatase activity toward both tyrosine-phosphorylated myelin basic protein and phospho-DARPP-32, with a much larger effect being observed for PP1[Y272A] than for PP1[Y272F].

Regulation of PP1/PP2A Chimeras by Protein Inhibitors and Targeting Subunits. Whereas the catalytic domains of PP1 and PP2A exhibit a high level of amino acid identity, there are significant differences in their C-terminal subdomains. PP1 and PP2A share the sequence FSAPNYC at the N-terminal half of the $\beta12/\beta13$ loop, and then their C termini diverge (Fig. 3). A chimeric protein, in which the PP2A sequence following the FSAPNYC region replaced the corresponding C terminus of PP1, was expressed in *E. coli* and was found, like PP2A, to exhibit markedly reduced sensitivity to phospho-inhibitor-1 and inhibitor-2 (ref. 22; see also ref. 30). The PP1/PP2A chimera expressed in Sf9 cells exhibited a large decrease in sensitivity to thiophospho-DARPP-32 and inhibitor-2, although both inhibitors were effective at concentrations of 1 μM and above (Fig. 1 and Table 1). Addition of a short peptide encompassing the docking motif of PNUTS [PNUTS (392–408)] (Y. G. Kwon, T.W., P.B.A., M. Kanarsha, P.G., and A.C.N., unpublished results), antagonized effectively the inhibition of wild-type Sf9 PP1 by either thiophospho-DARPP-32 or inhibitor-2, but had no effect on the inhibition of the Sf9 PP1/PP2A chimera seen at high concentrations of the inhibitors. Direct binding studies using GST-spinophilin (298–817) or PNUTS (309–691) showed that the Sf9 PP1/PP2A chimera did not interact with either protein (Fig. 2*A*). In addition, the Sf9 PP1/PP2A chimera was not inhibited by GST-spinophilin (298–817) or GST-PNUTS (309–691) (Fig. 2 *B* and *C*). Finally, the Sf9 PP1/PP2A chimera did not dephosphorylate phospho-DARPP-32 or tyrosine-phosphorylated myelin basic protein (Table 2).

Following the FSAPNYC sequence, three short regions of PP1—GEFD (residues 274–277), MC (residues 290–291), and QILK (residues 294–297)—are substituted with the sequences YRCG, KY, and LQFD, respectively, of PP2A. To further investigate the specificity of the interaction of protein inhibitors and targeting subunits with PP1, we replaced each of the three short regions in PP1 with the corresponding residues from PP2A. In addition, we replaced the three corresponding regions of the PP1/PP2A chimera with the residues found normally in PP1. Replacement of the MC sequence in PP1 with KY from PP2A resulted in a large increase in the IC_{50} for thiophospho-DARPP-32 (Fig. 4*A* and Table 1). However, replacement of QILK with LQFD, or of GEFD with YRCG, had no or little effect, respectively, on inhibition by thiophospho-DARPP-32. Conversely, replacement of the KY residues in the PP1/PP2A chimera with the MC residues found in PP1 largely restored the sensitivity to inhibition by thiophospho-DARPP-32 (Fig. 4*B*). The other two reverse mutations, LQFD to QILK or YRCG to GEFD, in the chimera had no effect. With respect to regulation by inhibitor-2, a qualitatively different pattern was observed for these three sets of mutants. Mutation of GEFD, MC, or QILK to the corresponding PP2A residues all resulted in a large increase in the IC_{50} for inhibitor-2 (Fig. 5 and Table 1). Moreover, none of the reverse mutations of the chimera restored sensitivity to inhibitor-2.

Discussion

Recombinant PP1, expressed in *E. coli*, has been used by our laboratory and several other laboratories to characterize the

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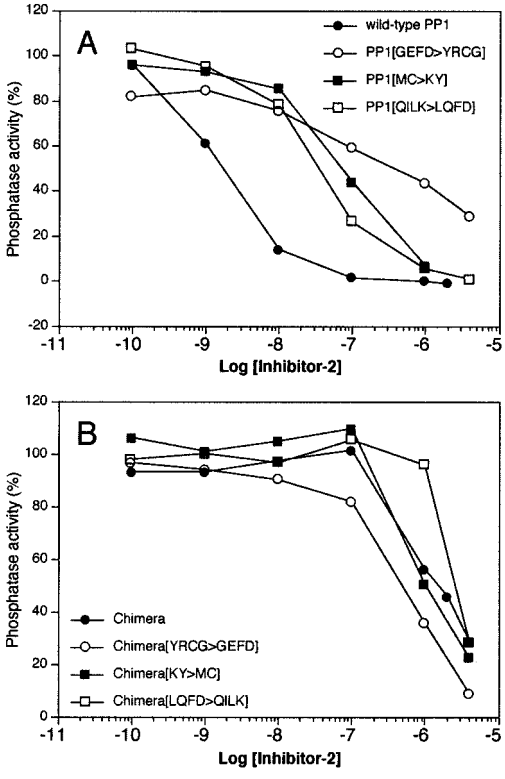

Fig. 5. Comparison of the regulation of PP1 and PP1/PP2A chimeras by inhibitor-2. Phosphatase activity was measured in the presence of the indicated concentrations of inhibitor-2. (*A*) Inhibition of wild-type PP1 and C-terminal domain mutants derived from PP1 as indicated. (*B*) Inhibition of PP1/PP2A chimera and mutants derived from the chimera as indicated. Phosphatase activity was measured as described in the Fig. 1 legend.

structure and regulation of the enzyme (20, 21, 23). However, *E. coli* PP1 exhibits enzymological properties that are very different from native PP1 (16, 20, 24, 25), and therefore, only limited conclusions have been drawn from those studies. In the present study, we have found that PP1 expressed in Sf9 insect cells exhibited properties essentially identical to those of native rabbit muscle PP1, making Sf9 PP1 a more appropriate preparation to use in detailed structure–function analyses.

The crystal structures of PP1 and the related phosphatase, PP2B, indicate that this class of enzymes contains two metal atoms embedded at the core of a conserved phosphoesterase motif (28, 29, 31). In the recombinant *E. coli* PP1 used for crystallization, Fe^{2+} and Mn^{2+} most likely are the two metals. Expression and purification of *E. coli* PP1 requires addition of Mn^{2+} to the growth media and purification buffers, and the purified enzyme is variably dependent on added Mn^{2+}, most likely because of the easy exchange of Mn^{2+} from the active site. Whereas the identity of the two metals in native PP1 remains unknown [studies of PP2A and PP2B suggest Fe^{2+} and Zn^{2+} are the likely candidates (31, 32)], the enzyme is fully active in the absence of added metals. In this regard, Sf9 PP1 was also fully active in the absence of added metals. This feature of native and Sf9 PP1 presumably occurs as a result of tighter binding of metals by these two preparations because of subtle changes in the geometry of the active-site metal-binding ligands.

Two crystal structures of *E. coli* PP1 have been determined, one of which represents an active form of the enzyme, and the other (in the presence of microcystin) represents an inhibited enzyme (28, 29). The two structures are essentially superimposable, the only difference being the position of the $\beta12/\beta13$ loop (22). Thus, the $\beta12/\beta13$ loop seems to be relatively flexible, and conceivably its incorrect positioning might be responsible for some of the different properties of *E. coli* PP1. In particular, the exact position of Y272 in the $\beta12/\beta13$ loop may be critical for the enzymatic properties of PP1. Y272 is conserved in PP1 and all related PPases, and its hydroxyl oxygen is positioned close to one of the active-site metals, making a hydrogen bond with an active-site water molecule (28). However, mutation of Y272 in *E. coli* PP1 had no effect on enzyme activity, although this mutation did affect the sensitivity to toxins, phospho-DARPP-32, and inhibitor-2 (19–21). In contrast, mutation of Y272 in Sf9 PP1 markedly decreased phosphatase activity, particularly when substituted with alanine. Moreover, whereas activity toward serine-phosphorylated phosphorylase *a* was decreased, the relative activity toward tyrosine-phosphorylated myelin basic protein was increased. Together these results suggest that the correct orientation of the hydrophobic phenol ring of Y272, situated near the active site, may be sufficient to block access of bulky tyrosine-phosphorylated substrates to the active site of native and Sf9 PP1 (and related serine/threonine PPases).

Our previous studies of DARPP-32 (and related studies of inhibitor-1) have indicated that phosphorylation of T34 (or T35) by cAMP-dependent protein kinase is essential for inhibition of PP1 (5, 21). It is likely that phospho-T34 is positioned close to or in the active site of PP1 in a manner in which it cannot be dephosphorylated, and thus it inhibits enzyme activity. The correct positioning of phospho-T34 may depend on the interaction of the docking motif of DARPP-32 (RKKIQF, residues 6–11) with the hydrophobic channel situated on the side of the enzyme opposite from that of the active site. Additional contacts between DARPP-32 and other parts of the C-terminal subdomain of PP1 are also likely. On the basis of initial modeling studies, we previously suggested that several acidic residues found in the acidic groove of PP1 might interact with the four arginine residues that precede phospho-T34 in DARPP-32 (28). However, mutation of these residues in various combinations in Sf9 PP1 had no effect on inhibition by thiophospho-DARPP-32. Subsequent studies have also shown that mutation of R29 and R30 in DARPP-32 did not affect its inhibitory properties (5). Together, these studies suggest that it is unlikely that DARPP-32 interacts with the acidic groove of PP1. Paradoxical results in which mutation of some acidic groove residues in *E. coli* PP1 actually increased the sensitivity to phospho-DARPP-32 or phospho-inhibitor-1 (20, 21) presumably reflected nonspecific changes in active site geometry. It remains possible that the acidic groove interacts with other PP1-binding proteins or substrates. Evidence in support of the latter possibility was obtained from the PP1[E252;D253A:E256R] mutant, which exhibited an increased apparent K_m for phosphorylase *a*.

In the absence of any interaction with the acidic groove, alternative modes for the binding of phospho-DARPP-32 or phospho-inhibitor-1 to PP1 could include their interaction with the C-terminal groove and/or interaction with the $\beta12/\beta13$ loop. Previous studies of (presumably) native PP1 using yeast two-hybrid analysis have indicated that mutations in A268, P269, A278, and G279 in the $\beta12/\beta13$ loop abrogated PP1 binding and inhibition by phospho-inhibitor-1 (21). The present study indicates that Y272 is also necessary for inhibition of PP1 by phospho-DARPP-32. Interestingly, mutation of Y272 to phenylalanine or alanine increased the ability of these mutant proteins to dephosphorylate both a phospho-tyrosine substrate and phospho-DARPP-32. These results provide additional support for the idea that phospho-T34 of DARPP-32 is situated close to or in the active site of native PP1 in a manner that makes it resistant to dephosphorylation. A268, P269, and Y272 are conserved in PP2A; therefore, it is unlikely that these specific amino acids mediate the PP1/phospho-inhibitor interaction. Rather, the overall structure of the $\beta12/\beta13$ loop may establish

the correct conformation of phospho-T34 of DARPP-32 (or phospho-T35 of inhibitor-1) at or near the active site of PP1.

Results obtained from studies of the PP1/PP2A chimera, as well as other previous structural studies (14, 20, 22, 30), indicate that PP1-specific residues (C-terminal to C273) are likely to be responsible for the binding of inhibitors and targeting subunits to PP1 and subsequent regulation of enzyme activity. The sequences GEFD (residues 274–277), MC (residues 290–291), and QILK (residues 294–297) are unique to PP1 and may play important roles in these interactions. The present study indicates that M290 and C291 are the key residues involved in the regulation of PP1 by thiophospho-DARPP-32. M290/C291 are located at the beginning of the β14 strand of PP1 and, together with L243, F257, and F293, help form the hydrophobic channel that interacts with the docking motif of the G_M peptide (RRVSFA, residues 64′-69′) (14). Specifically, C291 makes hydrophobic interactions with V66′ and F68′, hydrophilic interactions with S67′ and A69′, and M291 makes a hydrophobic interaction with A69′. Thus, a specific contribution of M290 and C291 to the binding of the docking motif of DARPP-32 would have been predicted. However, assuming that F11 of DARPP-32 interacts with the hydrophobic channel of PP1 in the same way as F68 of the G_M peptide, continued contributions from L243, F257, and F293 would have been expected. Given the size of the effect of this limited substitution of M290 and C291 on inhibition by thiophospho-DARPP-32, it is possible that subtle differences might exist in the binding of the docking motif of the phospho-inhibitors and the G_M peptide. Finally, the lack of effect of mutation of the GEFD and QILK residues on inhibition by thiophospho-DARPP-32 may provide information about the potential interaction(s) that the remainder of the inhibitory domain of DARPP-32 makes with PP1.

In contrast to the results obtained with thiophospho-DARPP-32, mutation of each of the three sets of PP1-specific residues influenced inhibition by inhibitor-2 to a similar extent, and the reverse mutations in the PP1/PP2A chimera had little effect. Although inhibitor-2 lacks a canonical PP1-docking motif, results from the present study, as well as previous studies (5, 13), showed that a peptide containing the PP1-docking motif [e.g., the PNUTS (392–408) peptide in this study] antagonized the ability of inhibitor-2 to inhibit PP1. These effects may be indirect and reflect an effect on the association of the unique N-terminal region of inhibitor-2 with a region of PP1 adjacent to the hydrophobic channel (18). Alternatively, inhibitor-2 may interact in an extended fashion with each of the three PP1-specific sequences, and mutation of any single region in the PP1/PP2A chimera may be insufficient to restore regulation of PP1 by inhibitor-2. In either case, these results further support the idea that the interaction of inhibitor-2 with PP1 is distinct from that of phospho-DARPP-32. These results also reinforce the idea that differences exist in the interaction of the hydrophobic channel with the various PP1-regulatory proteins, and that the mutant PP1 proteins analyzed in this study might be useful tools in physiological studies of PP1 function.

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