Importance of a Surface Hydrophobic Pocket on Protein Phosphatase-1 Catalytic Subunit in Recognizing Cellular Regulators*

Jennifer A. Gibbons‡, Douglas C. Weiser, and Shirish Shenolikar§

From the Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

Cellular functions of protein phosphatase-1 (PP1), a major eukaryotic serine/threonine phosphatase, are defined by the association of PP1 catalytic subunits with endogenous protein inhibitors and regulatory subunits. Many PP1 regulators share a consensus RVXF motif, which docks within a hydrophobic pocket on the surface of the PP1 catalytic subunit. Although these regulatory proteins also possess additional PP1-binding sites, mutations of the RV motif established a key role of this PP1-binding sequence in the function of PP1 regulators. WT PP1α, the C-terminal truncated PP1α-(1–306), a chimeric PP1α containing C-terminal sequences from PP2A, another phosphatase, PP1α-(1–306) with the RVXF-binding pocket substitutions L289R, M290K, and C291R, and PP2A were analyzed for their regulation by several mammalian proteins. These studies established that modifications of the RVXF-binding pocket had modest effects on the catalytic activity of PP1, as judged by recognition of substrates and sensitivity to toxins. However, the selected modifications impaired the sensitivity of PP1 to the inhibitor proteins, inhibitor-1 and inhibitor-2. In addition, they impaired the ability of PP1 to bind neurabin-I, the neuronal regulatory subunit, and G_m, the skeletal muscle glycogen-targeting subunit. These data suggested that differences in RVXF interactions with the hydrophobic pocket dictate the affinity of PP1 for cellular regulators. Substitution of a distinct RVXF sequence in inhibitor-1 that enhanced its binding and potency as a PP1 inhibitor emphasized the importance of the RVXF sequence in defining the function of this and other PP1 regulators. Our studies suggest that the diversity of RVXF sequences provides for dynamic physiological regulation of PP1 functions in eukaryotic cells.

Early studies by Ingebritsen and Cohen (1, 2) classified mammalian protein serine/threonine phosphatases into two major groups, type-1 and type-2, based in large part on their sensitivity to two endogenous protein inhibitors, inhibitor-1 (I-1) and inhibitor-2 (I-2). Type-I protein serine/threonine phosphatase or protein phosphatase-1 (PP1), displays unique and potent inhibition by nanomolar concentrations of I-1 and I-2. In contrast, PP2A and other type-2 protein phosphatases are essentially resistant to these inhibitor proteins. To date, more than 50 PP1-interacting proteins have been identified (3). Some, like I-1 and I-2, function as PP1 inhibitors, whereas others represent targeting subunits that direct subcellular localization and substrate recognition by multiprotein complexes containing PP1 catalytic subunits. Remarkably, PP1 and PP2A catalytic subunits share nearly 50% primary sequence identity, which may account for their overlapping substrate specificity in vitro (1). However, all PP1 regulators thus far analyzed demonstrate the unique ability displayed by I-1 and I-2 to selectively associate with PP1 and modify its catalytic function.

Detailed structure-function analyses of I-1 (4) and its neuronal homologue, DARPP-32 (5), first highlighted the tetrapeptide sequence KIQF, which acts in conjunction with PKA phosphorylation at a conserved threonine to inhibit PP1 activity. Subsequent studies noted a homologous sequence, RVSF, in the skeletal muscle glycogen-targeting subunit, G_m, that was also required for PP1 binding (6). Co-crystallization of PP1 with a synthetic dodecapeptide encompassing the RVSF sequence from G_m established the RVXF motif as a conserved PP1-binding sequence that associates with a hydrophobic pocket on the surface of the PP1 catalytic subunit (7).

There are extensive surface interactions between PP1 and regulatory subunits, as demonstrated by alanine-scanning mutagenesis of yeast PP1 (8) and the recently resolved structure of PP1 catalytic subunit complexed with the myosin-targeting subunit, MYPT1 (9). However, the ability of RVXF-containing peptides derived from several PP1 regulatory subunits to displace G_m from the glycogen-bound PP1 complex suggested a critical role for the RVXF sequence in PP1 binding (7). Single amino acid substitutions (e.g. Phe to Ala) in the RVXF motif severely impaired or abolished the ability of PP1 regulatory subunits (10) and inhibitors (4) to bind and regulate PP1. This emphasized the pivotal role played by the RVXF motif in PP1 binding and regulation. Several studies have identified RVXF-containing PP1-binding proteins using an overlay or far-Western with an isolated PP1 catalytic subunit (11, 12). Although this technique successfully identified many regulators, the pro-
otopic PP1 regulators I-1 and I-2 were either weakly or not detected using this technique. This suggested that not all RVXF-containing sequences were equivalent in PP1 binding. Direct binding studies also indicated that the unphosphorylated forms of I-1 (4) and DARPP-32 (5) bind PP1 very weakly. Although PKA phosphorylation activates these proteins as nanomolar inhibitors of PP1, the covalent modification only modestly increased their affinity for the PP1 catalytic subunit. Comparison of RVXF-containing synthetic peptides modeled on I-1 and Gm in their ability to disrupt neuronal PP1 complexes (13) also highlighted their differing affinities for PP1. Substituting the PP1-binding motif from nuclear inhibitor of PP1, RVTTF, in place of the KiQF sequence normally found in I-1 enhanced its potency as a PP1 inhibitor, and emphasized the key role played by the RVXF sequence in defining the function of PP1 regulators (12). These studies suggested that the differences in the association of this conserved sequence with the common binding site on the PP1 catalytic subunit defined its physiological regulation on cellular PP1 regulators. Our earlier studies (14) that substituted C-terminal sequences from PP2A in PP1α highlighted the diminished ability of the chimeric phosphatase, CHRM2, to bind I-1, I-2, nuclear inhibitor of PP1, and Gm. Later studies (15) showed similar deficits in CHRM2 binding to two other PP1 regulators, PP1 nuclear targeting subunit and spinophilin. Together, these studies suggested that the C-terminal sequences unique to PP1α played a key role in PP1 binding and regulation by cellular proteins. Attempts to reverse these regulatory defects in CHRM2 by systematic substitution of PP1-specific sequences in the PP2A-derived C terminus highlighted a PP1-specific sequence, 280-GC291, that enhanced the potency of DARPP-32 as an inhibitor of the modified CHRM2 (15). These and other data suggested that Met290 and Cys291, which line the hydrophobic RVXF-binding pocket and interact with the RVXF sequences in Gm (7) and MYPT1 (9), played a key role in PP1 regulation by cellular regulators. However, substitution of 280-GC291 in CHRM2 had little effect on its decreased inhibition by I-2. This suggested that the hydrophobic pocket on the PP1 catalytic subunit could distinguish RVXF motifs present in different regulators and thereby differentiate the ability of these proteins to regulate PP1 activity. Current studies deleted or substituted C-terminal sequences in PP1α as well as specifically modifying selected amino acids in the hydrophobic pocket of PP1α to define the role of the surface hydrophobic pocket in the recognition of RVXF-containing proteins. Our studies provided the first direct experimental evidence that the affinity of cellular regulators for the PP1 catalytic subunit is dictated by the RVXF sequence present in these proteins, and that the docking of this RVXF sequence in the surface hydrophobic pocket is conserved in all PP1 isoforms. Implications of these findings for the physiological regulation of PP1 in eukaryotic cells will be discussed.

**EXPERIMENTAL PROCEDURES**

**Materials—**Phosphorylase b was purchased from Calzyme, and phosphorylase kinase was obtained from Invitrogen. Anti-PP1 monoclonal antibody was obtained from Santa Cruz Biotechnology and horseradish peroxidase-conjugated anti-digoxigenin antibody was from US Biological. [γ-32P]ATP (3000 Ci/mmol) was purchased from Amersham Biosciences. Heparin-agarose was purchased from Bio-Rad. Digoxigenin-NHS (N-hydroxysuccinimide) was obtained from Roche Applied Science. N-Hydroxysuccinimide-activated Sepharose (Amersham Biosciences) was used to make microcystin-LR-Sepharose (MCLR-Sepharose) (16), and glutathione-Sepharose was obtained from Amersham Biosciences. MCLR and okadaic acid were purchased from Alexis Corp. Bacterial expression vector, pKK223-3, containing a cDNA encoding human PP1α (1-306) (PP1306) with additional seven C-terminal residues derived from the plasmid sequences, and the PP1α/PP2Aα chimeric subunit, CHRM2 (17), were provided by Richard Honkanen (University of South Alabama). The expression plasmid pTACTAC for full-length human PP1α (18) was obtained from Ernest Y.C. Lee (New York Medical College). Purified PP2A catalytic subunit (19) was provided by Brian Wadzinski (Vanderbilt University). The plasmid pGEX-2T containing the cDNA-encoding GST-I-1 was previously described (4), and GST-I-1 (20) was from Mathieu Bollen (Katholieke Universiteit, Leuven). The GST-88d-inhibitor-2 plasmid (20) was from Anna DePaoli-Roach (Indiana University). The expression plasmid encoding GST-Gm (1-240) (10) was provided by David L. Braunig (University of Virginia), and GST-neurabin-1 (374–516) was previously described (21).

**Preparation of Recombinant PP1α Catalytic Subunits—**All PP1α catalytic subunits were expressed in Escherichia coli BL21 grown overnight in Luria Broth containing 1 mM MnCl2 and 50 μM 2-mercaptoethanol (Sigma). Protein expression was induced by addition of 50 μM isopropyl-β-D-thiogalactopyranoside (Gold Technologies), with continued growth of the bacteria at 18 °C until Abs600 of culture was 0.6–0.8. Bacteria were sedimented at 4,000 × g for 10 min and resuspended in 50 mM Tris-HCl, pH 7.5, containing 0.1% (v/v) 2-mercaptoethanol, 1 mM EDTA, 1 mM MnCl2, and 10% (v/v) glycerol. Bacteria containing the protease inhibitors 1 mM benzamidine, 1 mM benzamidine, and 0.005% (v/v) glycercine were lysed using French pressure (−19,000 p.s.i.), and cell debris was removed by centrifugation at 30,000 × g for 30 min. The cleared lysates were subjected to chromatography on either heparin-agarose or MCLR-Sepharose to isolate PP1 catalytic subunits. For purification on heparin-agarose, bacterial extract was applied to the column and washed extensively with Buffer A containing 0.1 × NaCl. PP1 catalytic subunits were eluted with Buffer A containing 0.5 × NaCl. Fractons were collected and assayed for PP1 activity. Fractions were pooled, dialyzed overnight against Buffer A, concentrated using Centricon-10 (Pall Life Sciences), and dialyzed extensively against Buffer A containing 55% (v/v) glycerol prior to storage at −20 °C. Purification of PP1 catalytic subunits on MCLR-Sepharose was undertaken essentially as described by Moorhead et al. (16), and stored as described above.

**Preparation of Recombinant PP1 Regulators—**GST-I-1 fusion proteins were expressed, and phosphorylated as described (22), with the following modifications: bacteria were lysed by two passages through French press at −19,000 p.s.i. The fusion proteins were purified via glutathione-Sepharose according to manufacturer (Amersham Biosciences). GST-neurabin-1 (374–516) and GST-Gm (1–240) were expressed and purified as described (21). I-1 (23) and I-2 (12) were expressed and purified as previously described.

**32P-Phosphoprotein Substrates—**For phosphorylase a, phosphorylase kinase (0.25 mg) was added to a solution of phosphorylase b (10 mg/ml) in 100 mM β-glycerophosphate and 100 mM Tris-HCl, pH 8.2, containing 1 mM MgCl2, 100 μM ATP, 0.2 mM CaCl2, 0.1% (v/v) 2-mercaptoethanol, and 250–300 μCi of [γ-32P]ATP, and incubated at 37 °C. At 30-min intervals, aliquots (10 μl) of the reaction mixture were removed and added to 200 μl of 20% (v/v) trichloroacetic acid and 50 μl of bovine serum albumin (10 mg/ml). The sample was placed on ice for 2 min, then centrifuged at 15,000 × g for 10 min. The pellet was repeatedly washed with 20% (v/v) trichloroacetic acid prior to Cerenkov counting. Once the 32P incorporation reached a plateau (−90 min), the reaction was stopped by the addition of an equal volume of 90% saturated ammonium sulfate and placed on ice for 20 min. The mixture was centrifuged at 20,000 × g for 20 min, and the sedimented protein was washed twice with ice-cold 45% saturated ammonium sulfate. The pellet was then resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1 M NaCl, 0.1% (v/v) 2-mercaptoethanol, and dialyzed extensively against the same buffer.

For myelin basic protein (MBP), 200 μg of MBP was added to a solution of 1 mM ATP, 10 mM MgCl2, 10–20 μl of [γ-32P]ATP, and 50 mM Tris-HCl, pH 7.5. Then, 10–20 μl of purified PAKa was added to the reaction and incubated at 37 °C. 32P Incorporation was examined in the same manner as phosphorylase a, and as the 32P incorporation reached a plateau, the reaction was placed in dialysis buffer (50 mM Tris-HCl,
pH 7.5, 1 mM EDTA, and 0.1% (v/v) 2-mercaptoethanol, with frequent changes until background 32P diminished.

**Protein Phosphatase Assays**—PP1 catalytic subunits diluted in 50 mM Tris-HCl, pH 7.5, containing 1 mM MnCl2, 0.1% (v/v) 2-mercaptoethanol, and 1 mg/ml bovine serum albumin were incubated with [32P]Phosphorylase a (2 mg/ml) at 37 °C for 10 min (total reaction volume of 60 µl). Reaction was terminated by addition of 200 µl of 20% (v/v) trichloroacetic acid and 50 µl of bovine serum albumin (10 mg/ml), and the mixture was centrifuged at 15,000 × g for 5 min. [32P]Phosphate released into the supernatant was measured by liquid scintillation counting. Assays using MBP as substrate were performed in the following manner, using 10 µM MBP per reaction.

To assay PP1 inhibition by I-1, recombinant I-1 was first incubated with 0.3 mg/ml PKA, 50 mM Tris-HCl, pH 7.5, 0.2 mM ATP, and 2 mM MgCl2 at 30 °C for 72 h. The thio-phosphorylated I-1 was dialyzed into 50 mM Tris-HCl, pH 7.5, 0.005% (v/v) Brij-35, and 0.1% (v/v) 2-mercaptoethanol. Thio-phosphorylated I-1 was briefly incubated with PP1 before the addition of phosphorylation α. In assays for PP1 inhibition by I-2, the I-2 protein was preincubated with PP1 for 20 min at 37 °C, and targeting subunits were preincubated with PP1 for 5 min at 37 °C prior the initiation of the phosphatase assay.

**PP1 Sedimentation**—GST fusions of PP1 inhibitors and/or targeting subunits were incubated with glutathione-Sepharose (25-µl bed volume) equilibrated in Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 1 h at 4 °C. The beads were washed twice with TBS, and incubated for 1 h at 4 °C with bovine serum albumin (1 mg/ml). They were then washed twice with TBS, and recombinant PP1 was added for 1 h at 4 °C. The beads were washed four times with NETN-250 (250 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, and 0.5% (v/v) Nonidet P-40). Bound proteins were eluted with SDS sample buffer, and subjected to SDS-PAGE on 12% (w/v) polyacrylamide gels. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes, which were stained with Ponceau S to visualize the proteins. Membranes were blocked in 4% milk (w/v) in TBS plus 0.05% Tween, and PP1 detected by immunoblotting with an anti-PP1 antibody. The protein bands were quantified by scanning using Quantity One software (Bio-Rad).

**PP1 Far-Western Blots**—PP1 overlays were performed as described (11), with the following modifications. Protein staining using Ponceau S verified equivalent protein loading. The bound digoxigenin-labeled PP1 was detected by immunoblotting with an anti-digoxigenin antibody (horseradish peroxidase-linked) and ECL reaction (PerkinElmer Life Sciences). To ensure linearity of the overlay assays in each case, the ECL reaction was exposed to film for 2, 15, and 30 s.

For these assays, GST-T1 was phosphorylated using PKA (0.2 µg/ml), 100 µM ATP, 1 mM MgCl2, at 37 °C for 90 min. Phospho-GST-I-1 was dialyzed into 50 mM Tris-HCl, pH 7.5, 0.005% (v/v) Brij-35, and 0.1% (v/v) 2-mercaptoethanol. GST was similarly phosphorylated using PKA for control pull-down assays. PP1 binding to GST alone was defined as nonspecific and subtracted from that bound to GST fusion proteins containing known PP1 regulators. Protein concentration was determined using the Bio-Rad protein assay with BSA (Pierce) as the standard.

**Analysis of PP1 Structure**—The program Rasmol 2.6 (24) was used to generate the PP1 structure, as well as the programs Mage and Prekin (25), and interaction dots were generated with Molprobity (26) and Probe (27). The latter four programs were obtained from kinemage.biochem.duke.edu.

**RESULTS**

**Purification of Recombinant PP1 Catalytic Subunits**—Emerging studies (21, 28, 29) showed that PP1 regulators displayed selectivity for distinct PP1 isoforms, which differed largely in their C-terminal sequences. Prior studies (30, 31) suggested that extensive deletions of C-terminal sequences impaired or destabilized PP1α activity. Although recombinant PP1α-(1–297) demonstrated phosphorylase phosphatase activity equivalent to that of full-length PP1α, PP1α-(1–276) was not expressed in bacteria. To focus on the role of the RVX-binding site conserved in all PP1 isoforms, we expressed PP1α-(1–306) (PP1306), which eliminated the majority of C-terminal residues unique to this PP1 isoform. We also analyzed PP1α and CHRM2, which contains PP1α-(1–273) fused to the C-terminal 43 amino acids from bovine PP2Aα catalytic subunit, previously shown to generate an active phosphorylase phosphatase that was resistant to many PP1 regulators (14). In addition, we expressed PP1306 with the single amino acid substitutions L289R, M290K, and C291R to modify the RVX-binding pocket, which introduced residues more commonly found in type-2 phosphatases.

Previous studies utilized heparin-agarose to separate PP1 from type-2 protein serine/threonine phosphatases (32) and purify recombinant PP1 catalytic subunit expressed in E. coli (18). Complete purification of PP1, however, required multiple chromatographic steps and reduced the yields of this protein (18). Comparison of a recombinant PP1 catalytic subunit, such as CHRM2 (Fig. 1B), purified from bacterial extracts using either heparin-agarose or affinity chromatography with MCLR immobilized on Sepharose (16), demonstrated that MCLR-Sepharose yielded an essentially single polypeptide (>95% purity) as judged by SDS-PAGE (Fig. 1B, lane 3). By comparison, chromatography on heparin-agarose (Fig. 1B, lane 2), although eliminating >90% of bacterial proteins, yielded a preparation of CHRM2 that still contained other proteins. MCLR (IC50 < 1
C-terminal sequences in PP1306 (MCLR IC50 ble I). Compared with PP1, PP1
sensitivity of mutant catalytic subunits was either equal to or higher
A major share of the potent inhibitors was bound in the C-terminal region of PP1 catalytic subunits. Thus, PP1306, which retains the
activity against both substrates. In contrast, CHRM2 showed
the PP1 catalytic subunit (35–37). Thus, sensitivity of mutant
to okadaic acid compared with either PP1
or PP1306 (Table I and Fig. 2A). MCLR is an equipotent inhibitor of PP1 and PP2A. In contrast, okadaic acid demonstrates an IC50 for PP2A, which is ~100-fold lower than that for
PP1 (38). These data confirmed that the β12-β13 loop played a key role in defining the sensitivity of PP1 to okadaic acid (14).
Thus, PP1306, which retains the β12-β13 loop of PP1α, was inhibited by okadaic acid in a manner indistinguishable from
PP1α (Fig. 2A). By comparison, CHRM2, which incorporates the β12-β13 loop of PP2A, displayed nearly 10-fold greater sensitivity
to okadaic acid compared with either PP1α or PP1306. Further
introduction of L289R, M290K, or C291R in PP1306 had only a modest impact on the sensitivity of PP1 catalytic subunits to okadaic acid. Essentially identical results were obtained with

| PP1α | 157 ± 11 | 25 | 2 ± 0.1 | 1.75 ± 0.1 | 39 ± 1 | 28 ± 0.5 |
| PP1306 | 133 ± 23 | 29 ± 5 | 4.3 ± 0.5 | 7.3 ± 0.6 | 72 ± 4 | 40 ± 3 |
| L289R | 84 ± 31 | 66 ± 13 | 19 ± 6 | 380 ± 120 | 115 ± 15 | 76 ± 3 |
| M290K | 69 ± 21 | 52 ± 2 | 11 ± 0.5 | 157 ± 5 | 71 ± 6 | 28 ± 1 |
| C291R | 97 ± 29 | 285 ± 5 | 54 ± 11 | >1 μM | 158 ± 7 | 103* |
| CHRM2 | 18 ± 1 | 380* | 1,375 ± 675 | >1 μM |

* Assay was carried out once in duplicate.
teins have no effect on PP2A activity (4). In this regard, sensitivity of PP1306 to I-1 and I-2 was essentially identical to that of PP1α, indicating that the deletion of the C-terminal 24 amino acids had no effect on PP1 recognition by these protein regulators. However, as noted in previous studies (14), CHRM2 with the PP2A C terminus showed more than a 10-fold reduction in its IC₅₀ for I-1 and a nearly 500-fold reduced IC₅₀ for I-2 compared with either PP1α or PP1306 (Table I). This defect could result from the altered β₁β₂β₃ loop, previously shown to dictate sensitivity from PP1 to I-1 and I-2, or the absence of critical amino acids constituting the surface hydrophobic pocket that binds RVXF motifs present in I-1 (14, 23) and I-2 (39).

Although some modifications of the RVXF-binding pocket, L289R and M290K, showed modest decrements in IC₅₀ values for I-1 and I-2, C291R showed a dramatic loss in its sensitivity for I-1. The nearly 10-fold decrease in IC₅₀ for I-1 was almost equal to that seen with CHRM2 (Table I and Fig. 2B). This suggested that the substitution of a single amino acid, C291R, in PP1306 attenuated PP1 regulation by I-1 to the same extent as removal of 53 amino acids from the PP1α C terminus and their replacement by 42 amino acids of the PP2A C terminus, which generated CHRM2. This highlighted the critical importance of the surface hydrophobic pocket for PP1 regulation by I-1.

All three mutants also showed modest reductions in their sensitivity to I-2. In contrast to M290K and L289R, which showed a reduction of ~3- and 5-fold IC₅₀ for I-2, C291R demonstrated a >10-fold decrease in IC₅₀ for I-2. On the other hand, the reduction in I-2 sensitivity in all PP1306 mutants was significantly less than that seen in CHRM2 (Table I and Fig. 2C). This suggested differences in the mode of action of I-1 and I-2 as PP1 inhibitors. Although the fusion of PP2A C terminus significantly reduced the inhibition of CHRM2 by I-2 (IC₅₀ 1.35 μM), the chimeric phosphatase still retained some I-2 binding, consistent with previous studies that suggested that multiple regions of I-2 mediated PP1 binding (39). In contrast, the activity of PP2A was unaffected by 1 μM I-2 (Fig. 2C).

Association of WT and Mutant PP1α Catalytic Subunits with Regulatory Subunits—The diversity of RVXF sequences in PP1 regulators (3) and differences in various RVXF-containing peptides to disrupt cellular PP1 complexes (13) suggested that RVXF-containing PP1 regulators differ in their association with the surface hydrophobic pocket on PP1. To investigate PP1 binding to regulatory or targeting subunits with different RVXF sequences, we analyzed a recombinant G₅M, which contains the sequence RVSF (10), and a neurabin-I (Nrb) (21) polypeptide with the sequence KIKF; both sequences are required for PP1 binding. Full-length polypeptides, representing G₅M (1109 amino acids) and Nrb (1150 amino acids), are either poorly or not expressed in bacteria. Thus, we expressed GST-G₅M (1-240) and GST-Nrb (374–516), which contain the key elements required for PP1 binding.

The binding of WT and mutant PP1 catalytic subunits to GST-Nrb and GST-G₅M was analyzed by sedimentation of these complexes bound to glutathione-Sepharose. The presence of GST fusion proteins was analyzed by protein staining with Ponceau S, and PP1 was detected by immunoblotting with an anti-PP1 antibody. PP1306 binding to GST-Nrb and GST-G₅M is shown in Fig. 3A. With increasing concentrations of targeting subunit, increased amounts of PP1306 were sedimented. Binding to CHRM2, L289R, M290K, and C291R was analyzed in a similar manner, and the bound PP1 catalytic subunits were quantified by densitometry. Under the conditions of this assay, binding to PP1306 was essentially saturated at 5 and 10 μg of GST-G₅M (Fig. 3B). PP1α showed greater binding to GST-G₅M than PP1306, particularly at low concentrations (1 μg) of the GST fusion protein (data not shown). Prior studies (14) suggested that CHRM2 bound more weakly than PP1α to GST-G₅M (1-215). This difference was greatly magnified in our assays, with CHRM2 showing little or no binding to GST-G₅M (1-240). All three point mutants, L289R, M290K, and C291R, were compromised in their ability to bind GST-G₅M. Although M290K and L289R bound GST-G₅M weakly, C291R, like CHRM2, failed to bind this fusion protein at all concentrations analyzed (Fig. 3B). This suggested that, as noted with I-1, the hydrophobic pocket, particularly the amino acid C291, played a critical role in PP1 binding by G₅M.

Surprisingly, under conditions that GST-G₅M failed to bind CHRM2, GST-Nrb bound CHRM2 weakly, requiring 10 μg of GST-Nrb to visualize significant CHRM2 binding (Fig. 3B). This was unexpected as prior studies (15) had demonstrated no binding of CHRM2 to much higher concentrations of GST-spinophilin, a structural homologue of neurabin. This could reflect the use of a different fragment of spinophilin/neurabin-II, namely amino acids 298–817, compared with residues 374–516 of neurabin-I used in this study. Alternatively, these differences could be attributed to the different assay conditions or some differences in the primary sequences of the PP1-binding site. Remarkably, unlike GST-G₅M, GST-Nrb bound both L289R and M290K almost as effectively as PP1306, indicating that these mutations did not totally disrupt the RVXF-binding
The C-terminal substitution C291R renders PP1306 insensitive to inhibition by neurabin-I. The dose-dependent inhibition of phosphorylase phosphatase activity of PP1306 and mutant phosphatases by GST-Nrb is shown. Representative curves for the inhibition of PP1306 (open circles), CHRM2 (closed circles), PP1306 C291R (open triangles), and PP2A (closed triangles) are shown.

In contrast to GM, which only partially inhibits the phosphorylase phosphatase activity of the PP1 catalytic subunit (10), Nrb is a potent PP1 inhibitor in the same assay (21). Thus, GST-Nrb inhibited PP1α with an IC₅₀ of ~2 nM (Fig. 4 and Table I). By comparison, PP2A was essentially insensitive to GST-Nrb at concentrations up to 1 μM (Fig. 4). PP1306 was inhibited by GST-Nrb with a modestly reduced IC₅₀ = 7.3 ± 0.6 nM. In contrast, neither CHRM2 nor C291R were inhibited by GST-Nrb at up to 1 μM concentration (Fig. 4). L289R and M290K showed ~50-fold and 15-fold reductions in IC₅₀ for GST-Nrb, respectively (Table I). Although these experiments emphasized that changes in PP1 binding were not quantitatively linked to the altered enzyme activity, mutations of the RVF-binding pocket had generally similar effects on both functions. For example, the weakened binding displayed by GST-Nrb for L289R and M290R led to 50- and 15-fold reduction in PP1 inhibitory activity. By contrast, C291R, which failed to bind GST-Nrb in pull-down assays, was unable to inhibit phosphorylase phosphatase activity of PP1 at concentrations up to 1 μM.

Role of the RVF Sequencing in I-1 Function—The above studies suggested that RVF sequences in various PP1 regulators bound differently within the surface hydrophobic pocket, and thus, were influenced variably by substitutions of different amino acids in the RVXF-binding pocket. To further test this hypothesis, we analyzed WT I-1KIQF and a mutant I-1RVTF. The mutant I-1RVTF was created by substitution of the RVTF sequence derived from nuclear inhibitor of PP1, a PP1-binding protein that can be visualized readily by overlays (12). We utilized an overlay assay in which PP1-binding proteins were subjected to SDS-PAGE, and following electrophoretic transfer to polyvinylidene difluoride membranes, partially renatured. Incubation of membranes with soluble digoxigenin-conjugated PP1 catalytic subunits, followed by immunoblotting with anti-digoxigenin antibody, allowed for direct comparison of PP1 binding to several different target proteins. However, due to the denaturation-renaturation involved in PP1 overlays, this assay favored the detection of PP1 regulators containing RVXF motifs (21).

GST-Nrb and GST-GM both showed dose-dependent binding by digoxigenin-coupled PP1306 (Fig. 5). By comparison to GST-Nrb and GST-GM, the PKA-phosphorylated GST-I-1 (GST-I-1-P) bound PP1306 very weakly, requiring 10-fold higher protein for detectable PP1 binding. At similar exposures as Nrb and GM, PP1306 binding to GST-I-1-P was undetectable. Substitution of the RVTF sequence in GST-I-1 increased PP1306 binding to GST-I-1RVTF-P, albeit the mutant I-1 still bound PP1306 100-fold weaker than GST-GM. The unphosphorylated GST-I-1 and GST-I-1RVTF, like GST alone, failed to bind any of the PP1 catalytic subunits (data not shown).

The binding of PP1α and mutant PP1 catalytic subunits to several regulators is summarized in Table II. These data showed that, in overlays, PP1306 bound more effectively than PP1α to all regulators analyzed. As noted in pull-down assays, compared with PP1306, all three mutants, L289R, M290K, and C291R, showed reduced binding to GST-GM. C291R also bound GST-Nrb more weakly than either L289R or M290K. The already weak PP1 binding to GST-I-1-P was essentially abolished by all three mutations in the RVF-binding pocket. However, substitution of the KVTF sequence in I-1 allowed low but detectable binding by M290K. In general, results obtained with overlays (Table II) paralleled those seen with pull-down assays (Fig. 3). Together, these data suggested that not only the context, namely the parent PP1 regulator, but also the actual RVXF sequence played key roles in defining the affinity of PP1 for cellular regulators.

**DISCUSSION**

The primary structure of PP1 demonstrates >80% sequence identity from plants to animals, making PP1 one of the most highly conserved proteins in evolution (40). Consistent with its evolutionary conservation, PP1 regulates many critical functions in eukaryotic cells, including transcription, translation, metabolism, cell growth, and differentiation. Regardless of its species origin, PP1 can be readily distinguished from other protein serine/threonine phosphatases by its unique ability to be inhibited by the mammalian inhibitor proteins I-1 and I-2. Both of these PP1 regulators utilize multiple domains to bind and regulate PP1 activity. For example, in addition to the N-terminal KIQF sequence, PKA phosphorylation at threonine-35 is critical for PP1 inhibition by I-1 (4). More recent studies suggested that C-terminal sequences in I-1 also played a role in PP1 binding and regulation (41). Up to five different regions of the I-2 protein are thought to participate in PP1 regulation (39, 42). These and other studies have fostered the hypothesis that cellular regulators have evolved a combinatorial mechanism, utilizing multiple interaction domains, some of which are common to a subset of regulators, to modulate cellular PP1 functions.

Our prior studies utilized a number of different ways to identify the structural determinants on the PP1 catalytic subunit that defined its regulation by cellular proteins. Such studies included the analysis of random mutations (23), surface "charged-to-alanine" substitutions (42), a “core” PP1 catalytic
Interactions of PP1 with Cellular Regulators

Comparison of WT and mutant PP1 catalytic subunits binding to mammalian PP1 regulators

PP1 binding was analyzed using overlays as described under “Experimental Procedures,” and binding was assessed by chemiluminescence. In three independent experiments, PP1 binding as seen by chemiluminescence was analyzed by exposure of the overlays to film for 2, 15, and 90 s.

| GST-Nrb | GST-G_M | GST-I-1-P | GST-I-1-KVP-P | GST-P |
|---------|---------|-----------|---------------|-------|
| PP1α    | ++*     | +         | -             | +/-   | -     |
| PP1306  | +++     | +++       | +/-           | +     | -     |
| L289R   | ++      | +/-       | -             | -/-   | -     |
| M290K   | ++      | +/-       | -             | -/-   | -     |
| C291N   | +/-     | +/-       | -             | -/-   | -     |
| CHRM2   | +/-     | +/-       | -             | -/-   | -     |

*++ denotes a dark band seen in 15 s; +++ denotes a dark band seen within 2 s; + indicates a dark band in 30 s; +/- denotes a faint band seen in 30 s; and – denotes no bands seen after prolonged exposure.

Figure 6. Association of the RVXF sequence with the hydrophobic pocket on the PP1 catalytic subunit. The structure of PP1γ1 bound to a synthetic peptide (representing amino acids 63–75) encompassing the RVSF sequence from the skeletal muscle glycogen-targeting subunit GM is shown (7). A, PP1 is shown in space-fill model using Rasmol. The G_M peptide is shown as a stick model in red. The amino acids that comprise the RVXF-binding pocket are shown in blue, with residues modified in this study (L289, M290, and C291) shown in cyan. B shows the interactions of Leu^{289} (red), Met^{290} (green), and Cys^{291} (purple) with the G_M peptide using the Mage program. The G_M peptide is shown in red, and the amino acids lining the RVXF-binding pocket are shown in gray with cyan side chains. The strength of interactions of the RVSF sequence is displayed as dots generated using the Probe program and illustrate associations with Leu^{289} (red), Met^{290} (green), and Cys^{291} (purple).

Subunit (amino acids 41–269) that lacked the N- and C-terminal sequences unique to specific PP1 isoforms and a chimeric PP1α (CHRM2), in which the C-terminal PP1 sequences were substituted with those from PP2A (14). Together, these studies highlighted the β12-β13 loop (amino acids 269–282) overhanging the catalytic site as a key determinant defining the sensitivity of PP1 to selected toxins and endogenous PP1 inhibitors, specifically I-1 and I-2. Comparative studies of PP1α and CHRM2 also suggested that the C terminus played a role in the affinity of PP1 for targeting subunits such as G_M (14), PP1 nuclear targeting subunit and spinophilin (15), all of which either bound poorly or failed to bind CHRM2. These results were confirmed in the current studies, which emphasized the significant decrements in CHRM2 binding to not only G_M but also neurabin and I-1.

The C-terminal residues of the PP1 catalytic subunit also contribute to the formation of a surface hydrophobic pocket that binds the RVXF motif conserved in many PP1 regulators (7). Prior studies noted that substitution of the sequence 290-291, which lines the hydrophobic pocket, impaired PP1 regulation by DARPP-32 and to a much lesser extent, I-2 (15). Some PP1 regulators also displayed a remarkable selectivity for distinct PP1 isoforms. Thus, G_M selectively bound PP1β in skeletal muscle (29) and neuronal neurabin complexes primarily contained PP1γ1 (21, 29). While the mechanism underlying PP1 isoform selectivity of regulators is not fully understood, it most likely reflects the divergent C-terminal sequences found in the different PP1 isoforms. To specifically focus on the functional analysis of the RVXF-binding pocket that is conserved in all PP1 isoforms, we undertook a limited C-terminal deletion of human PP1α to yield PP1306, which lacked the PP1α-specific sequences. PP1306 was efficiently expressed in E. coli, and rapidly purified using one-step affinity chromatography with MCLR-Sepharose. The purified PP1306 showed increased enzyme activity against both phosphorylase a and MBP when compared with PP1α. This was consistent with earlier studies, which showed that limited proteolysis of the 37-kDa PP1 catalytic subunit yielded a 35-kDa polypeptide with increased phosphorylase phosphatase activity (43). These studies suggested that the extreme C terminus of PP1α modulated its enzymatic activity.

Previous studies (44) that undertook modifications of the surface hydrophobic pocket in the single yeast PP1 catalytic subunit showed that alanine substitutions of two or more amino acids in this pocket induced lethality in yeast. Many of the “lethal” PP1 catalytic subunits, assayed as immunoprecipitates, displayed significant MBP phosphatase activity. Interestingly, yeast lethality correlated with near complete loss of
phosphorylase phosphatase activity of the mutant PP1 catalytic subunits. As regulatory subunits modify the substrate specificity of the bound PP1 (3), these data suggested the inability of mutant PP1 catalytic subunits to bind one or more regulators required for PP1-catalyzed events essential for yeast viability. To investigate the role of the hydrophobic pocket in recognition of PP1 regulators, we introduced single substitutions in the 289LMC291 sequence that lined the RVXF-binding pocket in PP1306, substituting amino acids more commonly found in type-2 phosphatases (40). Specifically, Leu289 was replaced with Arg found in a plant PP2A (45), Met290 was substituted with Lys present in mammalian PP2A and C291 with Arg found in PP2B and several PP2A-like phosphatases. The high phosphorylase α and MBP phosphatase activity of all mutant PP1306 enzymes showed that these substitutions did not alter their catalytic function. This was further verified by demonstrating that the toxin sensitivity of the PP1306 mutants was similar to that of PP1α.

C291R resulted in deficits in PP1 inhibition by I-1 and Nrb (Table I and Fig. 4) comparable to those seen in CHRM2, in which 53 PP1α-specific C-terminal residues were replaced by 42 amino acids from the PP2A C terminus. In contrast, L289R and M290K had intermediate effects on PP1306 regulation by the same proteins. Direct binding analyses using either PP1 pull-down assays or overlays confirmed the critical importance of Cys291 in binding Nrb, I-1, and GmM. PP1 pull-down assays also showed that, whereas both L289R and M290K had modest effects on Nrb binding, M290K, and particularly L289R, were significantly impaired in their ability to bind GmM. Interestingly, in yeast (44), the substitution C290A, corresponding to human Cys291, bound GAC1, the yeast homologue of GmM effectively, and accumulated WW levels of glycogen. In contrast, L289A bound GAC1 poorly, and displayed a low glycogen phenotype. Whether these differences reflect the distinct RVXF motifs present in mammalian GmM (RV5F) and yeast GAC1 (KNVRF), or the differing amino acid substitutions analyzed, remains unknown, but both L289A and C290A bound other yeast PP1 regulators, Reg1 and Sds22, like WT Glc7 (44). These data pointed to differences in the association of various PP1 regulators with the RVXF-binding pocket.

Our data highlighted three C-terminal amino acids, L288, M290, and C291, which bind the highly extended RVXF sequences (Fig. 6). Cys291 and Leu289 are particularly important, because Cys291 makes direct contacts with the phenylalanine that is conserved in nearly all RVXF-containing PP1-binding proteins, and Leu289 interacts with the valine or isoleucine also found in most PP1 regulators. Interestingly, x-ray crystallography of p53bp2, a known PP1-binding protein, suggested that the region encompassing the PP1-binding sequence, RVKF, is isosteric, but a similar substitution of RVTF in place of the proposed surface hydrophobic pocket in PP1 association with the targeting subunit MYPT1 confirmed the critical role played by the surface hydrophobic pocket on the PP1β catalytic subunit (9). Comparison of the flanking sequences surrounding the RVXF motifs in MYPT1 and I-1 suggested that these sequences followed distinct paths across the rear surface of the PP1 catalytic subunit to approach the catalytic site on the opposing surface and thus elicit their distinct functional effects on PP1 activity (9). Recent studies also suggested that the N- (21) and C-terminal (29) sequences flanking the RVXF motif account for the selectivity of some regulators for distinct PP1 isoforms.

To establish the critical role played by the RVXF sequence in defining the affinity of PP1 for cellular regulators, we analyzed WT GST-I-1 and the mutant GST-I-1RVTF (12). The mutant I-1RVTF showed a >10-fold increased binding to PP1306 (Fig. 5) and was also a 3- to 5-fold more potent inhibitor than WT I-1 (12). The identity of the RVXF motif in I-2 remains controversial, but a similar substitution of RVTF in place of the proposed PP1-binding sequence, KLHY, abolished the ability of I-2 to inhibit PP1 holoenzymes. This demonstrated that the substitution of the same RVXF motif has opposing functional effects on I-1 and I-2 and further hinted that, compared with the known RVXF-containing regulators, GmM, Nrb, and I-1, I-2 utilized a distinct mode of PP1 binding. On other hand, our previous studies (42) showed that an IKGI sequence in I-2 bound at a site adjacent to the hydrophobic pocket and, thus, I-2 binding was indirectly influenced by the substitutions L289R, M290K, and C291R. None of the pocket mutants elicited the dramatic loss in I-2 sensitivity seen in CHRM2, which was at least 200-fold less sensitive. This also provided evidence that I-2 differed from GmM, Nrb, and I-1 in its association with PP1.

The current studies demonstrated the critical role played by the surface hydrophobic pocket in PP1 association with the three cellular regulators GmM, Nrb, and I-1. They also highlighted the differing affinities of RVXF-containing PP1 regulators for the PP1α catalytic subunit, which may result from differences in the binding of distinct RVXF sequences with the surface hydrophobic pocket on the PP1 catalytic subunit. This diversity in PP1 interactions with different RVXF-containing regulators may be an important evolutionary strategy that allows further regulation of PP1 by physiological signals. Thus, the weak PP1 binding displayed by I-1, although enhanced by its phosphorylation by PKA, may be insufficient to displace more tightly bound PP1 regulatory subunits, such as GmM and Nrb, and thus in the absence of other modifications, I-1 is unable to inhibit PP1 complexes containing these regulators. In this regard, it is worth noting that PKA also phosphorlyates serines within or adjacent to the RVF motifs in GmM (47) and Nrb (48) to attenuate their association with the PP1 catalytic subunit. This in turn may facilitate the regulation of these cellular PP1 complexes by I-1 in response to hormones that elevate cAMP. Alternately, additional interactions between I-1 and regulatory subunits, such as the growth arrest and DNA damage-inducible protein GADD34 (49), may be required to circumvent the competition of these two RVXF-containing proteins for a common site on the surface of the PP1 catalytic subunit and still permit I-1 to transduce the hormonal signals that regulate the PP1/GADD34 complex and eukaryotic protein translation (41, 49). In conclusion, further studies are clearly needed to investigate the contribution of all residues that make up the RVXF-binding pocket, and thus gain a full understanding of the role of the hydrophobic pocket in the binding of cellular regulators to distinct PP1 isoforms. However, our data point to a novel experimental strategy directed at modifying or eliminating PP1 association with selected proteins and thereby elucidate the physiological role of specific PP1 complexes.

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