Importance of the \( \beta_{12}-\beta_{13} \) Loop in Protein Phosphatase-1 Catalytic Subunit for Inhibition by Toxins and Mammalian Protein Inhibitors*

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Type-1 protein serine/threonine phosphatases (PP1) are uniquely inhibited by the mammalian proteins, inhibitor-1 (I-1), inhibitor-2 (I-2), and nuclear inhibitor of PP1 (NIPP-1). In addition, several natural compounds inhibit both PP1 and the type-2 phosphatase, PP2A. Deletion of C-terminal sequences that included the \( \beta_{12}-\beta_{13} \) loop attenuated the inhibition of the resulting PP1α catalytic core by I-1, I-2, NIPP-1, and several toxins, including tautomycin, microcystin-LR, calyculin A, and okadaic acid. Substitution of C-terminal sequences from the PP2A catalytic subunit produced a chimeric enzyme, CRHM2, that was inhibited by toxins with dose-response characteristics of PP1 and not PP2A. However, CRHM2 was insensitive to the PP1-specific inhibitors, I-1, I-2, and NIPP-1. The anticancer compound, fostriecin, differed from other phosphatase inhibitors in that it inhibited wild-type PP1α, the PP1α catalytic core, and CRHM2 with identical IC₅₀. Binding of wild-type and mutant phosphatases to immobilized microcystin-LR, NIPP-1, and I-2 established that the \( \beta_{12}-\beta_{13} \) loop was essential for the association of PP1 with toxins and the protein inhibitors. These studies point to the importance of the \( \beta_{12}-\beta_{13} \) loop structure and conformation for the control of PP1 functions by toxins and endogenous proteins.

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‡ The abbreviations used are: PP1, protein phosphatase-1; PP2A, protein phosphatase-2A; I-1, inhibitor-1; DARPP-32, dopamine- and cAMP-regulated phosphoprotein of apparent Mᵦ 32,000; I-2, inhibitor-2; NIPP-1, nuclear inhibitor of PP1; CRHM2, a chimera of PP11-273 and PP2A267-309; GST, glutathione S-transferase; WT, wild-type.
importance of the $\beta_12-\beta_13$ loop in PP1 catalytic subunit

tions in the yeast calcineurin or PP2B catalytic subunit that converted Thr-350 in the $\beta_12-\beta_13$ loop to either lysine or arginine and reduced its sensitivity to inhibition by the natural product and immunosuppressive drug cyclosporin (13). Together, these data point to the $\beta_12-\beta_13$ loop as a common site for inhibitory mechanisms that impinge on this family of protein serine/threonine phosphatases.

The precise contribution of the $\beta_12-\beta_13$ loop in phosphatase inhibition by structurally diverse endogenous inhibitors and natural compounds remains unknown. Thus, we undertook a detailed biochemical analysis of a PP1α catalytic subunit from which the $\beta_12-\beta_13$ loop had been deleted and a chimeric PP1α catalytic subunit that incorporated the $\beta_12-\beta_13$ loop and C-terminal sequences from a different serine/threonine phosphatase, PP2A. These studies established the absolute requirement of the $\beta_12-\beta_13$ loop structure for PP1 inhibition by many different inhibitors and suggested that the toxins and endogenous protein inhibitors recognized distinct C-terminal sequences. The role of the $\beta_12-\beta_13$ loop in binding phosphatase inhibitors and the potential role of conformation changes in this structure in PP1 inhibition are discussed.

MATERIALS AND METHODS

Tautomycin, microcystin-LR, okadaic acid, and calyculin A were purchased from Calbiochem. Phosphorylase kinase and phosphorylase $b$ were obtained from Life Technologies, Inc. (γ-$\gamma$-PIATP was purchased from Amersham Pharmacia Biotech. The digoxigenin-labeling kit was obtained from Roche Molecular Biochemicals. 1-2 and NIPP-1 (recombinant central domain) were kindly provided by Mathieu Bollen (Catholic University of Leuven, Belgium), I-2-Sepharose was provided by Ernest Y. C. Lee (New York Medical College). A bacterial expression vector for GST-G5, the N-terminal 215 amino acids of human skeletal muscle glycogen-targeting subunit, $G_{\alpha}$, fused to glutathione S-transferase was provided by David L. Bruggaert (University of Virginia, Charlottesville). The PP2A catalytic subunit purified from bovine brain was obtained from Brian Wadzinski of Vanderbilt University Medical School. Fosfriecin was kindly provided by Parke-Davis.

Protein Purification—Recombinant human PP1α and CRHM2, a chimeric consisting of residues 1–273 of human PP1α fused in-frame to residues 267–309 from the bovine PP2A catalytic subunit, were expressed as described by Walsh et al. (13). Briefly, pKX225–2 containing the appropriate cDNA was transformed into Escherichia coli JM105. The bacteria were grown in LB medium containing 1 mM MnCl$_2$ and 50 µM isopropyl-$\beta$-thiogalactopyranoside for 48 h or until the absorbance at 600 nm was $\sim$0.6. The bacteria were sedimented by centrifugation, resuspended in 0.001 volume of 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 0.1% (w/v) Nonidet P-40, 0.1% (v/v) β-mercaptoethanol, and lysed by passing twice through the French press. The lysate was cleared by centrifugation (25 min at 15,000 $\times$ g). The supernatant was adjusted to 20% (v/v) glycerol and applied to heparin-Sepharose. The column was washed with 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 50 mM NaCl, 20% (v/v) glycerol, and 0.1% (v/v) β-mercaptoethanol. The PP1 catalytic subunit was eluted with the same buffer containing 500 mM NaCl. Fractions containing phosphorylase phosphatase activity were pooled and dialyzed against 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 50 mM NaCl, 20% (v/v) glycerol, and 0.1% (v/v) β-mercaptoethanol before loading on to a second heparin-Sepharose column. This time, the phosphatase was eluted using a linear gradient of the same buffer containing 50–500 mM NaCl. Both WT PP1 and CRHM2 were eluted from this column between 300 and 400 mM NaCl. Fractions containing PP1 activity were pooled, concentrated using Centricon-10, and further purified by gel filtration on Sephadex 200. This yielded a highly purified PP1 catalytic subunit represented by a 37-kDa polypeptide that accounted for $\sim$90% of the total protein.

The PP1α catalytic core (residues 41–269) was expressed in bacteria and purified as described previously (15). Recombinant human inhibitor-1 was expressed, purified, and phosphorylated as described by Connor et al. (16). GST-G5 was produced according to Wu et al. (17).

Phosphorylase Phosphatase Assays—Protein phosphatase activity was assayed by the release of $^{32}$P phosphate from phosphorylase $a$ as described by Shenolikar and Ingelfinger (18). The recombinant phosphatases were incubated with 10 µM phosphorylase $a$ in 50 mM Tris-HCl, pH 7.0, 1 mg/ml bovine serum albumin, 1 mM MnCl$_2$, 0.3% (v/v) β-mercaptoethanol (total volume 60 µl) at 37 °C for 10 min. The reaction was terminated by the addition of 0.2 ml of 20% (w/v) trichloroacetic acid and 50 µl of bovine serum albumin (6–10 mg/ml). Following centrifugation at 15,000 $\times$ g for 5 min, the supernatant (200 µl) was analyzed for $^{32}$P release by liquid scintillation counting. $^{32}$P release was calculated from the counts present in the assay. One unit of phosphorylase phosphatase activity is defined as releasing 0.2 nmoles of phosphate in 1 min in the standard assay.

In assays with toxins, I-1, and NIPP1, which inhibit PP1 activity almost instantaneously, the reaction was initiated by the addition of enzyme to the substrate/inhibitor mixture preincubated at 37 °C for 10 min. However, for I-2, which shows a time-dependent inhibition of enzyme activity, the enzyme and inhibitor were preincubated for 20 min at 37 °C to ensure maximal inhibition and the assay initiated by the addition of the radiolabeled substrate.

PP1 Association with Immobilized Phosphatase Inhibitors—PP1 binding to microcystin-LR-Sepharose (19), I-2-Sepharose (20), and NIPP-1-Sepharose (21) was carried out as described previously. Briefly, a 40-µl packed volume of affinity matrix was washed three times with 10 volumes of 50 mM Tris-HCl, pH 7.0, 1 mg/ml bovine serum albumin, 1 mM MnCl$_2$, and 0.3% (v/v) β-mercaptoethanol. The beads were then incubated in 200 µl of PP1 core, WT PP1α, or CRHM2 (10 units/ml) at 4 °C for 30 min. The beads were then pelleted by centrifugation, and the supernatant (20 µl) was assayed for residual PP1 activity using phosphorylase $a$ as substrate.

For Westerns with Digoxigenin-labeled PP1 and CRHM2—PP1α and CRHM2 were covalently modified using the digoxigenin labeling kit (Roche Molecular Biochemicals). The PP1-binding proteins were separated by 10% (w/v) polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) SDS and then electrophotographed transferred to the polyvinylidene difluoride membrane. The membrane was incubated with blocking solution containing 3% (w/v) dry milk in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, and the PP1-binding proteins were detected by incubation with digoxigenin-conjugated PP1α or CRHM2 followed by an anti-digoxigenin antibody as described by Jagiello et al. (8).

RESULTS

PP1 enzymes from many species, including rabbit (22), fly (23), and yeast (24), are all inhibited by nanomolar concentrations of rabbit muscle I-1 and I-2. This is consistent with the high degree of structural conservation seen in human PP1α (25), Drosophila 87B (26), and Saccharomyces cerevisiae GLC7 (27). The most significant differences in their primary sequences are restricted to their extreme N termini (3) and C-terminal sequences extending beyond the $\beta_12-\beta_13$ loop (Fig. 1A). To establish the importance of the $\beta_12-\beta_13$ loop in PP1 regulation, we expressed the PP1α "catalytic core," residues 41–269, which excluded the $\beta_12-\beta_13$ loop as well as the divergent N- and C-terminal sequences.

The C-terminal sequence in PP1α differs significantly from that of PP2A (3), which is characterized by its insensitivity to mammalian PP1 inhibitors. Yet, PP1 and PP2A share the sequence FSAPNYC, which constitutes the N-terminal half of mammalian PP1 inhibitors. Yet, PP1 and PP2A share the sequence FSAPNYC, which constitutes the N-terminal half of mammalian PP1 inhibitors. Yet, PP1 and PP2A share the sequence FSAPNYC, which constitutes the N-terminal half of mammalian PP1 inhibitors.
Importance of the β12-β13 Loop in PP1 Catalytic Subunit

**Fig. 1. Comparison of C-terminal sequences surrounding the β12-13 loop in PP1 and PP2A catalytic subunits.** A shows the C-terminal sequences in PP1 catalytic subunits from rabbit, Drosophila, and S. cerevisiae. The β12-β13 loop is indicated by a bar. The triangles indicate the position of the C-terminal deletion that yielded an active PP1α core. B shows the C-terminal sequences of human PP1α and bovine PP2A Cα catalytic subunit with the β12-β13 loop highlighted by a bar. Regions of amino acid identity in A and B are shown with a gray background, and conservative substitutions are marked by a lighter background. A dash indicates space inserted for optimal alignment of the amino acid sequences. C is a schematic of the enzymes used in this study with the catalytic core that is the most highly conserved in each class of enzymes represented by a wide bar with the variable N and C termini indicated as narrower bars. The PP1α core (residues 41–269) lacked the N- and C-terminal variable regions present in the WT PP1α catalytic subunit, whereas the chimera, CRHM2, consisted of N-terminal residues 1–273 of human PP1α fused to C-terminal residues 267–309 from bovine PP2A.

Unlike I-1, do not require phosphorylation to inhibit phosphatase activity. Wild-type human PP1α was potently inhibited by NIPP-1 and I-2 with the half-maximal concentrations at or below 1 nM. By comparison, the PP1α core was not inhibited by either protein at greater than 100-fold higher concentrations (data not shown).

Earlier studies (15) reported that the PP1α core was resistant to selected concentrations of the toxins, okadaic acid and microcystin-LR. More detailed dose-response curves established that WT PP1α was inhibited by microcystin-LR with an IC$_{50}$ of approximately 1 nM. In contrast, the PP1α core was not inhibited by microcystin-LR at concentrations exceeding 2 μM (data not shown). The PP1α core also showed a greater than 1000-fold reduction in its sensitivity to other xenobiotic compounds, including tautomycin and calyculin A (data not shown).

The antitumor compound, fostriecin, inhibits PP2A (14) and PP4 (28) at nanomolar concentrations but is a weaker PP1 inhibitor. Fostriecin inhibited wild-type PP1α and the catalytic core with identical IC$_{50}$ values of approximately 0.2 mM (Fig. 2A). This is similar to WT PP1α (Fig. 3B). Calyculin, which also shows a 10-fold preference as a PP1 inhibitor, also inhibited CRHM2 like WT PP1α with an IC$_{50}$ of approximately 0.1 nM (data not shown). Finally, we confirmed the findings of Walsh et al. (14) that fostriecin inhibited both WT PP1α and CRHM2 in an identical manner requiring high micromolar concentrations of the drug. By comparison, nanomolar concentrations of fostriecin inhibited PP2A activity. Thus, the sensitivity of CRHM2 to several different toxins was identical to WT PP1α and was therefore most likely defined by the N-terminal 271 residues.

**Inhibition of CRHM2 by Mammalian PP1 Inhibitors**—The PP1/PP2A chimera, CRHM2, was also analyzed for inhibition by three different mammalian PP1-specific inhibitors, I-1, I-2, and NIPP-1. As I-1 is only a PP1 inhibitor when phosphorylated by cAMP-dependent protein kinase, we utilized constitutively active thiophosphorylated I-1. This avoided the possibility that CRHM2 may acquire the ability of PP2A to dephosphorylate and inactivate I-1 in the assay. As previously noted (29), the recombinant human PP1α was less sensitive to inhibition by I-1, with an IC$_{50}$ of approximately 300 nM, than PP1 catalytic subunit purified rabbit skeletal muscle (IC$_{50}$ 1 nM). CRHM2 behaved more like PP2A than PP1 and was not inhibited by thiophosphorylated I-1 at several hundred-fold higher concentrations (data not shown).

On the other hand, I-2 inhibited WT PP1α with an IC$_{50}$ of approximately 1 nM (Fig. 4), a value that is essentially identical to that obtained with the native PP1 catalytic subunit isolated from mammalian tissues. NIPP-1 was also an equally potent inhibitor of recombinant PP1α and native PP1 catalytic subunits (data not shown) with an IC$_{50}$ below 1 nM. However, neither I-2 (Fig. 4) nor NIPP-1 (data not shown) inhibited CRHM2 activity at several hundred-fold higher concentrations. Thus, in contrast to toxins, where CRHM2 largely demonstrated the properties of PP1α, the presence of PP2A C-termin-
nal sequences severely impaired CRHM2 inhibition by the mammalian PP1 inhibitors, making it more like PP2A.

PP1 Binding to Immobilized Phosphatase Inhibitors—Early studies showed that PP1 was proteolyzed in muscle extracts to yield a 35-kDa rather than 37-kDa catalytic subunit, which lacked C-terminal sequences and was destabilized in its association with I-2 (30). To evaluate the contribution of PP1 C-terminal sequences eliminated in the PP1α catalytic core or substituted with PP2A C-terminal sequences in CRHM2, in its association with phosphatase inhibitors, we analyzed the direct binding of these enzymes to microcystin-LR, NIPP-1, I-2, and thiophosphorylated I-1 immobilized to Sepharose.

Consistent with the inhibition of WT PP1α and CRHM2 by microcystin-LR, both activities were readily adsorbed on microcystin-LR-Sepharose, which removed more than 98% of the proteins from solution (Fig. 5A). The enzyme binding to the affinity matrix was confirmed by immunoblot analysis using an anti-PP1 monoclonal antibody (data not shown) following their release in SDS-sample buffer. The PP1α core, which was insensitive to microcystin-LR, failed to bind the immobilized toxin. Greater than 95% of enzyme activity remained in solution even after prolonged incubation with microcystin-LR-Sepharose.

The immobilized NIPP-1, I-2, and thiophosphorylated I-1 all effectively adsorbed WT PP1α, removing between 70 and 90% of enzyme activity, as expected by the ability of these proteins to inhibit enzyme activity (Fig. 5A). Of the three affinity matrices, thiophosphorylated I-1-Sepharose was the least effective in depleting PP1 activity, perhaps reflecting the loss in affinity of recombinant PP1 catalytic subunits for I-1 discussed above (29, 31). To our surprise, CRHM2, which was insensitive to the three protein inhibitors, bound all affinity matrices, albeit with slightly reduced efficacy when compared with WT PP1α. The decreased binding of CRHM2 was most notable with thiophosphorylated I-1-Sepharose. The PP1α core failed to bind any of the immobilized protein inhibitors, as expected given the in-

![Figure 2](image2.png)

**Fig. 2.** Inhibition of the PP1α catalytic core by the anticancer compound, fostriecin. A, purified bovine PP2A catalytic subunit (open triangles), recombinant WT human PP1α (open squares), and the PP1α catalytic core (closed diamonds) were assayed for phosphorylase phosphatase activity in the presence of increasing concentrations of fostriecin. Representative results from one of three independent experiments carried out in duplicate are shown. B, wild-type PP1α was assayed for phosphorylase phosphatase activity in the presence of increasing concentrations of microcystin-LR in the absence (open squares) and in the presence (closed diamonds) of 0.2 mM fostriecin. The PP1α activity in the presence of 0.2 mM fostriecin alone was reduced by approximately 45%.

![Figure 3](image3.png)

**Fig. 3.** Inhibition of the PP1/PP2A chimera, CRHM2, by toxins. A shows the inhibition of phosphorylase phosphatase activity of wild-type PP1α (open squares) and CRHM2 (closed diamonds) by microcystin-LR. B shows the inhibition of WT PP1α (open squares), CRHM2 (closed diamonds), and purified bovine PP2A catalytic subunit (open triangles) by tautomycin.

![Figure 4](image4.png)

**Fig. 4.** Inhibition of CRHM2 by inhibitor-2. Inhibition of WT PP1α (open squares) and CRHM2 (closed diamonds) by I-2, a well characterized PP1-specific inhibitor, is shown. A representative result from three independent experiments is shown with each value representing the average of three assays with standard error of less than 5%.
ability of the mammalian PP1 inhibitors to inhibit the mutant enzyme even at very high concentrations.

**CRHM2 Binds to RVXF-containing PP1 Regulators**—I-1 (22), I-2 (7), and NIPP-1 (21) possess multiple sites of interaction with the PP1 catalytic subunit. One key interaction is mediated through a conserved tetrapeptide (RVXF) motif present in many other PP1 regulators. Co-crystallization of the PP1 catalytic subunit with a synthetic peptide from the skeletal muscle glycogen-targeting subunit, G\textsubscript{M}, identified the RVXF-binding pocket (32). Comparison of PP1\textalpha and CRHM2 predicted a substitution, cysteine 291 to tyrosine, within the RVXF-binding pocket in the chimeric enzyme, which could account for the insensitivity of CRHM2 to I-1, I-2, and NIPP-1 and its slightly reduced binding to the immobilized inhibitors. Thus, we analyzed PP1 binding to G\textsubscript{M}, whose sequence first defined the RVXF-binding pocket using a far western assay with digoxigenin-derivated WT PP1\textalpha and CRHM2. This assay identified numerous PP1-binding proteins containing the conserved motif (19) and appears to principally monitor the association of PP1 with the RVXF motif (21). WT PP1\textalpha and CRHM2 bound a range of concentrations of GST-G\textsubscript{M} in an identical manner (Fig. 5B). Substitution of two key residues, Val and Phe, within the RVXF motif with Ala abolished PP1\textalpha binding to GST-G\textsubscript{M} (data not shown), confirming that the association was mediated through the RVXF sequence. Thus, the several hundred-fold reduced sensitivity of CRHM2 to I-1, I-2, and NIPP-1 was not attributable to their diminished binding in the RVXF-binding pocket.

**DISCUSSION**

Structure-function studies of four mammalian PP1 inhibitors, I-1 (22), DARPP-32 (33), I-2 (7), and NIPP-1 (21), established that multiple domains in these proteins are required to inhibit PP1 activity. However, the cognate regions of PP1 catalytic subunit recognized by the inhibitors remained unknown. I-1 and DARPP-32 are structural homologues, which share little sequence homology with I-2 or NIPP-1. The sole common feature in the four PP1 inhibitors is a tetrapeptide sequence known as the RVXF motif. Deletion of this sequence inactivates I-1 (22) and DARPP-32 (33) as PP1 inhibitors, suggesting that the motif is important for enzyme inhibition. However, synthetic peptides lacking the RVXF sequence modeled on NIPP-1 were as effective PP1 inhibitors as peptides containing the motif. This proved that the RVXF motif was not essential for PP1 inhibition by NIPP-1 (21). RVXF sequences have also been identified in many PP1-binding proteins that are not inhibitors of this enzyme. These proteins target PP1 to various subcellular organelles and modify its substrate specificity. Although RVXF-containing peptides derived from many different targeting subunits displace PP1 from these regulators (32), they do not inhibit PP1. Indeed, high concentrations of the M\textsubscript{110}\textsubscript{10}-(1–38) peptide derived from the myosin-targeting subunit enhanced PP1 activity toward myosin light chain (34). Co-crystallization of a synthetic G\textsubscript{M} peptide localized RVXF binding to a surface of the PP1 catalytic subunit diagonally opposite to the catalytic site (32). This also made it difficult to predict the functional impact of the RVXF binding on PP1 activity. Thus, we proposed that the RVXF motif represents an anchoring motif that defines the specificity of PP1 regulators (29), and its association may position other functional domains in these regulators to bind and modify PP1 activity.

We recently identified a domain in the PP1 catalytic subunit, the \beta12-\beta13 loop, that may be particularly important for its association with inhibitors. Deletion of this domain abrogated PP1 inhibition by phosphorylated I-1 but had no notable impact on the recognition of substrates by the enzyme (10). Specific residues in this loop were also implicated in PP1 inhibition by toxins (20), and the PP1\textalpha catalytic core, which lacked the \beta12-\beta13 loop, was insensitive to okadaic acid and microcystin-LR (15). We confirmed and extended these findings to include toxins like tautomycin and calyculin A, which showed a preference for PP1 over other protein serine/threonine phosphatases. Inability of microcystin-LR to inhibit the PP1\textalpha catalytic core is particularly interesting as this toxin has been co-crystallized with PP1\textalpha, and its location within the PP1 catalytic site has been clearly defined (35). Microcystin-LR shows a remarkable fit within the PP1 catalytic site, with tight packing of the Adda (3-amino-9-methoxy-2,6,8-trimethyldeca-4,6-dienoic acid) side chain in a hydrophobic groove emanating from the catalytic center, association with the catalytic metals through water molecules, and close proximity of the unusual N-methyldehydroalanine residue with the \beta12-\beta13 loop where it forms a covalent adduct with Cys-273. However, substituting Cys-273 with other residues established that adduct formation was not essential for PP1 inhibition (36). On the other hand, mutation of the adjacent Tyr-272 (20) or deletion of the entire \beta12-\beta13 loop, as shown here, severely impaired PP1 inhibition by microcystin-LR and other toxins. This suggests that the loop is critical for enzyme inhibition. Molecular modeling of the PP1...
catalytic site (37) suggests that several other toxins associate with tyrosine 272 and adjacent residues in the β12-β13 loop. Thus, our demonstration that the PP1α core failed to bind to microcystin-LR-Sepharose for the first time established that the β12-β13 loop is absolutely required for both toxin binding and enzyme inhibition.

Zhang et al. (38) exchanged a short sequence, GEFD, in the β12-β13 loop of PP1α with YRCG found in PP2A. This substitution slightly increased the sensitivity of the chimeric PP1α to inhibition by okadaic acid, suggesting that this region of the loop accounted for the higher sensitivity of PP2A to the toxin. CRHM2, which contained a much larger portion of PP2A C terminus including the YRCG sequence, was even closer to PP2A in its sensitivity to okadaic acid, suggesting that additional C-terminal sequences also contributed to its okadaic acid sensitivity. On the other hand, CRHM2 was inhibited by many other toxins in a manner identical to WT PP1α, suggesting that the PP2A sequences contributed little to the affinity of CRHM2 for these compounds. Interestingly, the N-terminal half of the β12-β13 loop represented by the sequence, FSAPNY, is conserved in all members of this enzyme family including PP2B (calcineurin), which is essentially resistant to all the toxins that we analyzed. So we speculated that the β12-β13 loop structure may be required for toxin binding, but other regions of the PP1 and PP2A catalytic sites define their inhibition by the toxins. We attempted to address this by a finer dissection of the β12-β13 loop. However, C-terminal truncations of the PP1α catalytic subunit, terminating at either Phe-276 or Tyr-272 failed to yield active phosphatases, as noted by others (39). So the precise function of the β12-β13 loop remains unknown.

PP1 inhibition by the mammalian proteins, I-1, I-2, and NIPP-1, involves multiple interactions between these proteins and the phosphatase catalytic subunit that extend beyond the catalytic site occupied by the small molecular weight toxins. We have already discussed the RVXF-binding pocket that lies on the opposite surface of the PP1 catalytic subunit from the catalytic center and perhaps defines the specificity of I-1, I-2, and NIPP-1 as PP1 regulators. Thus, it is remarkable that the elimination of the β12-β13 loop that produced the catalytic core abolished PP1 inhibition by I-1 (10), I-2, and NIPP-1. An equally surprising finding was that the PP1α core failed to bind all three of these inhibitors immobilized on Sepharose. This emphasized the critical importance of the β12-β13 loop in the binding of PP1 to the protein inhibitors. It is interesting to note that PP1α is often purified from mammalian tissues as a 35-kDa partially proteolyzed catalytic subunit that lacks the C-terminal sequences. This enzyme is inhibited by nanomolar concentrations of I-2 like the full-length 37-kDa PP1 catalytic subunit. Both catalytic subunits form stable inactive PP1-I-2 complexes that are fully reactivated by the phosphorylation of I-2 by GSK-3. However, unlike the stable active complex containing the 37-kDa catalytic subunit, GSK-3-mediated phosphorylation of I-2 results in its dissociation from the 35-kDa PP1. This suggests that the loss of C-terminal sequences destabilized the association of PP1 with I-2 (30). Comparing these data with the PP1α core, which lost both binding and inhibition by I-2, suggests that proteolysis in tissue extracts may retain the β12-β13 loop in PP1 required for its effective inhibition by I-2 but remove other C-terminal sequences that stabilize its association with I-2.

Precisely how PP1 inhibitors interact with the β12-β13 loop remains to be determined. The inability of all three mammalian proteins to inhibit the PP1α core (residues 41–269) combined with the conservation of primary sequences in different PP1 catalytic subunits (Fig. 1A) suggests that the critical elements for PP1 inhibition reside between residues 269 and 301.

Our previous studies identified several mutations (A269T, P270L, A279V, and G280S) at the base of the β12-β13 loop that were important for PP1 binding to I-1 in the yeast two-hybrid assay (10). In contrast, mutations of Tyr-272 near the middle of the loop only modestly impaired PP1α inhibition by I-1 (10) and I-2 (20). Moreover, this residue is present in CRHM2 and the type-2 protein serine/threonine phosphatases that are resistant to I-1, I-2, and NIPP-1. Thus, if the proteins interact with Tyr-272, it is clearly not sufficient for PP1 inhibition. In considering the importance of the primary sequence of the β12-β13 loop for PP1 inhibition by I-1, I-2, and NIPP-1, it should be noted that the chimeric enzyme, CRHM2, although not inhibited by these proteins, still bound the immobilized proteins. This was particularly notable with I-2 and NIPP-1. This indi-
icated that although the presence of a β12-β13 loop may account for CRHM2 ability to bind I-2 and other PP1 inhibitors, yet other PP1-specific sequences in the loop and C terminus (i.e., residues 269–301) define its sensitivity to the protein inhibitors. Comparison of PP1α and CRHM2 sequences between residues 269 and 301 (Fig. 1B) showed only 14 nonconservative substitutions with six of these being within the β12-β13 loop. Our earlier studies (10) exchanged four of these, GEFI (residues 274–278), for the YRCG sequence found in PP2A and found no effect of PP1 inhibition by I-1. Thus, we speculate that the lack of interaction with the toxin inhibitor. Thus, the ability of protein inhibitors to change the conformation of the β12-β13 loop may also be important for enzyme inhibition, and mutations within the loop or at its base may alter its flexibility and reduce the sensitivity of PP1 to inhibitors.

It is also clear from these experiments that the β12-β13 loop is not the sole determinant of PP1 inhibition by all inhibitors. We have already mentioned the particular importance of the RXVF motif for enzyme inhibition by I-1 and DARPP-32. In the present studies, we have shown that fostriecin, an anti-cancer compound, inhibits the PP1α core and CRHM2 with the same efficacy as WT PP1α, suggesting that it relies on domains other than the β12-β13 loop to suppress PP1 activity. It is tempting to speculate that the lack of interaction with the β12-β13 loop accounts for the reduced potency of fostriecin as a PP1 inhibitor. In any case, the antitumor activity of fostriecin occurs at concentrations that may inhibit PP1 and other serine/threonine phosphatases. This raises the intriguing possibility that elucidating the mode of PP1 inhibition by fostriecin may lead to a molecular strategy that can distinguish potential therapeutic compounds from natural products that are potent tumor promoters (40). Understanding the mechanism of action of phosphatase inhibitors may also yield experimental approaches to delineate the physiological importance of endogenous PP1 inhibitors in hormone signaling.

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