Inhibition of myosin phosphatase is critical for agonist-induced contractility of vascular smooth muscle. The protein CPI-17 is a phosphorylation-dependent inhibitor of myosin phosphatase and, in response to agonists, Thr-38 is phosphorylated by protein kinase C, producing a >1,000-fold increase in inhibitory potency. Here, we addressed how CPI-17 could selectively inhibit myosin phosphatase among other protein phosphatase-1 (PP1) holoenzymes. PP1 in cell lysates was separated by sequential affinity chromatography into at least two fractions, one bound specifically to thiophospho-CPI-17, and another bound specifically to inhibitor-2. The MYPT1 regulatory subunit of myosin phosphatase was concentrated only in the fraction bound to thiophospho-CPI-17. This binding was eliminated by addition of excess microcystin-LR to the lysate, showing that binding at the active site of PP1 is required. Phospho-CPI-17 failed to inhibit glycogen-bound PP1 from skeletal muscle, composed primarily of PP1 with the striated muscle glycogen-targeting subunit (Gα) regulatory subunit. Phospho-CPI-17 was dephosphorylated during assay of glycogen-bound PP1, not MYPT1-associated PP1, even though these two holoenzymes have the same PP1 catalytic subunit. Phosphorylation of CPI-17 in rabbit arteries was enhanced by calyculin A but not okadaic acid or fostriecin, consistent with PP1-mediated dephosphorylation. We propose that CPI-17 binds at the PP1 active site where it is dephosphorylated, but association of MYPT1 with PP1 allosterically retards this hydrolysis, resulting in formation of a complex of MYPT1-PP1C-P-CPI-17, leading to an increase in smooth muscle contraction.

Protein phosphatase-1 (PP1) is a dominant Ser/Thr phosphatase, controlling a plethora of events in cells, from yeast to mammals. Cellular PP1 holoenzymes consist of a catalytic subunit (PP1C) and various regulatory (targeting) subunits. PP1C in mammalian cell exists as four isoforms that contain a conserved catalytic domain plus a variable region in the C-terminal tails (1). On the other hand, >50 PP1 regulatory subunits have been discovered in mammalian cells (reviewed in refs. 2 and 3). These subunits have a common PP1-binding sequence motif, VxF, that associates with PP1C on the backside opposite the active site, tethering PP1C to compartmentalize PP1 activity (4). Additionally, the interaction with regulatory subunits results in allosteric modulation of PP1C substrate specificity (5). Thus, PP1 subunits modify properties of PP1C to generate diversity of function in cells (2, 3).

In addition to regulatory subunits, several PP1-specific inhibitor proteins are present in mammalian cells. These inhibitor proteins potently suppress the activity of purified PP1C at nanomolar concentrations (6). Most PP1 inhibitor proteins are phosphoproteins, suggesting that cellular PP1 activities are modulated in response to kinase signaling via phosphorylation of PP1 inhibitor proteins. Originally, PP1 inhibitor proteins such as inhibitor-1 (I-1) and inhibitor-2 (Inh2) were believed to inhibit only the free PP1C released from regulatory subunits but not the PP1 holoenzymes themselves. This concept was based on results showing that neither I-1 nor Inh2 blocked activity of the glycoinhibitor-1 (I-1) and inhibitor-2 (Inh2) were believed to inhibit glycogen-bound PP1 from skeletal muscle, composed primarily of PP1 with the striated muscle glycogen-targeting subunit (Gα) regulatory subunit. Phospho-CPI-17 was dephosphorylated during assay of glycogen-bound PP1, not MYPT1-associated PP1, even though these two holoenzymes have the same PP1 catalytic subunit. Phosphorylation of CPI-17 in rabbit arteries was enhanced by calyculin A but not okadaic acid or fostriecin, consistent with PP1-mediated dephosphorylation. We propose that CPI-17 binds at the PP1 active site where it is dephosphorylated, but association of MYPT1 with PP1 allosterically retards this hydrolysis, resulting in formation of a complex of MYPT1-PP1C-P-CPI-17, leading to an increase in smooth muscle contraction.

Experimental Procedures

Materials. Recombinant His-6, S-tag (H6S)-CPI-17, and (H6S)-Inh2 were prepared as described (6). Thiophosphorylation and phosphorylation were performed by using ATPγS (Roche Applied Science, Indianapolis) and ATP (Sigma), respectively. Antibodies for pan-PP1C and MYPT1 were purchased from Transduction Laboratories (Lexington, KY) and Babco (Richmond, CA), respectively. Anti-myc epitope (9E10) antibody was obtained from the Developmental Hybridoma Bank at the University of Iowa. Antibodies for catalytic subunit of PP2A (PP2Ac), CPI-17, and P-CPI-17(T38) were prepared as described (18, 22, 24). S-protein agarose and glutathione-agarose were purchased from Transduction Laboratories (Lexington, KY) and Babco (Richmond, CA), respectively. Anti-myc epitope (9E10) antibody was obtained from the Developmental Hybridoma Bank at the University of Iowa. Antibodies for catalytic subunit of PP2A (PP2Ac), CPI-17, and P-CPI-17(T38) were prepared as described (18, 22, 24). S-protein agarose and glutathione-agarose were purchased from Novagen and Sigma, respectively. Microcystin-LR (MC-LR) was obtained from Calbiochem and coupled with Affigel 10 (Bio-Rad, Hercules, CA) according to the manufacturer’s pro.
tocol. Preparation of the expression vector for myc epitope-tagged human MYPT1 (myc-MYPT1) was described previously (25). An Ala-mutant of myc-MYPT1 at Phe-38, F38A, was prepared by the QuikChange protocol of Stratagene. A PCR fragment encoding MYPT1 (1–300) was ligated into pGEX4T-2 at BamHI/EcoRI sites. The fusion protein, GST-MYPT1 (1–300), was expressed in Escherichia coli, by induction with isopropyl thio-
β-galactoside and purified by using glutathione-agarose chromatography. Myosin phosphatase was isolated from pig aorta (9). Glycogen particles from a rabbit skeletal muscle extract were prepared by the protocol of Grupposo and Braut
tigan (26). This preparation contains major glycogen-associating proteins such as glycogen phosphorylase and glycogen synthase (26), and has been used for starting material of glycogen-bound PP1 preparation (7). Cell culture materials were obtained from Invitrogen. COS7 and NIH 3T3 cells were obtained from American Type Culture Collection and maintained with 10% newborn calf serum in DMEM. Rat aorta smooth muscle cells were a generous gift from G. Owen's lab at the University of Virginia and were maintained with 10% FBS in DMEM/F12 (1:1) medium.

Binding Assay ("Pull-Down Assay"). Cells in a 10-cm dish were lysed with 1 ml of lysis buffer (50 mM 4-morpholinepropanesulfonic acid–NaOH, pH 7.0, including 0.1 M NaCl/1 mM EGTA/0.1% Triton X-100/5% glycerol/0.2% 2-mercaptoethanol/0.4 mM Pefabloc). After centrifugation at 20,000 × g for 10 min, an aliquot (0.25 ml) of the lysate was mixed for 30 min at 4°C with 5 μg of either H2S-CPI-17 or H2S-Inh2 immobilized on a slurry (10 μl) of S-protein agarose beads (Novagen). The beads were recovered, washed three times by centrifugation with 0.15 ml of lysis buffer, and mixed with 20 μl of 2× Laemmli buffer. Eluted proteins were immunoblotted with anti-MYPT1 (1:5,000 dilution), anti-PP1C (1:1,000) or anti-PP2Ac (1:5,000). Using these conditions, ~70% of total MYPT1 is recovered in the soluble fraction, as described (27).

Sequential Pull-Down Assay. Rat aorta smooth muscle cells in a 15-cm dish were lysed with 1 ml of lysis buffer. The extract clarified by centrifugation at 20,000 × g for 20 min was mixed for 3 h at 4°C with 5 μg of either unphosphorylated H2S-CPI-17 thio phosphorylated H2S-CPI-17, or H2S-Inh2 plus 10 μl of S-protein agarose slurry. These conditions were determined to be limiting for the amount of extracts so the inhibitors would fully deplete the sample of their binding partners. The suspension was centrifuged as before, and the supernatant was transferred into another tube. The beads collected, washed, and proteins eluted and analyzed as described above.

Immunoprecipitation Assay. COS7 cells in a 10-cm dish were transiently transfected for 24 h with myc-MYPT1 vector (5 μg) by using 0.1 μl of FuGENE6 transfection reagent (Roche Ap
plicated Sciences), as described (23). Cells expressing myc-MYPT1 were lysed with 1 ml of lysis buffer. The extract was clarified by centrifugation for 30 min at 10,000 × g and split into two portions. Half was incubated overnight with anti-myc antibody (4 μg) on ice. The anti-myc immunocomplex was captured for 30 min with 10 μl of Protein G-agarose slurry (Sigma) at 4°C. The other half of the extract was subjected to pull-down assay by using thiophospho-CPI-17 (TP-CPI-17) beads. After washing three times with 0.15 ml of lysis buffer, proteins on beads were solubilized by addition of Laemmli buffer, and the samples were subjected to immunoblotting analysis.

Phosphatase Assay. Phosphatase activity was measured by using [32P]-labeled substrate, 1 μM ATP/LC20 for myosin phosphatase, or 0.3 mg/ml [32P]-phosphatase a, for glycogen-bound phosphatase, as described (28). Conditions used were 25 mM 4-morpholino-
prolinepropanesulfonic acid–NaOH, pH 7.0, including 50 mM NaCl/0.1 mM EGTA/1 mM okadaic acid/1 mM DTT/0.02% Brij-35/0.4 mM Pefabloc. Caffeine (5 mM) was added to phos-
phorylase-a phosphatase assay. Reaction was initiated by addition of myosin phosphatase (3.9 microunits) or glycogen-bound phosphatase (56 microunits). Phosphorylation of Thr-38 in CPI-17 in the mixture was measured by immunoblotting by using anti-P-CPI-17(T38) (1:1,000). The blot was reprobed with anti-
CPI-17 (1:5,000) for a loading control.

Smooth Muscle Tissue Preparation and Measurement of CPI-17 Phos-
phorylation. All animal procedures were approved by the Animal Care and Use Committee of the Boston Biomedical Research Institute (Boston). Femoral artery strips from adult male New Zealand White rabbits were equipped on a transducer throughout experiments and were subjected to permeabilization with Staphylococcus aureus α-toxin (20 μg/ml; List, Campbell, CA), as described (29). Permeabilized strips were first treated with the ATP-free Ca2+-free solution for 30 min and then incubated for 20 min in the presence of phosphatase inhibitors with 30 μM GTP/Na. Phosphorylation was initiated by addition of 4.5 mM MgATP and 10 mM creatine phosphate at 20°C. After 4 min incubation, strips were quickly frozen and subjected to the immunoassay for phosphorylation of CPI-17. Phosphorylation of CPI-17 was measured by anti-P-CPI-17(T38) immunoblotting, as described (18).

Others. SDS/PAGE, immunoblotting, densitometry, and protein determination were carried out as described (6).

Results

Selective Binding of Inhibitor Proteins to PP1 Holoenzymes. Selective binding of PP1 holoenzymes to TP-CPI-17 and Inh2 was analyzed by a two-stage “sequential pull-down assay” (Fig. 1). In the first stage, a cell lysate was mixed with TP-CPI-17 or Inh2 coupled to beads. In the second stage, the unbound fractions from each pull down were split in half and subjected to a second pull-down assay with fresh batches of TP-CPI-17 and Inh2 beads. The amount of PP1 holoenzyme bound was estimated by immuno
blotting, using anti-PP1C antibody. In the first stage, substantial amounts of PP1C were bound to either TP-CPI-17 or Inh2, compared to control, where no PP1C was bound to unphosphorylated CPI-17 (Fig. 1A). Using the unbound fraction from TP-CPI-17 beads yielded little PP1C binding in the second stage with TP-CPI-17 beads, indicating this pool of PP1 holoenzymes was depleted in the first stage. However, this same unbound fraction had substantial amounts of PP1 holoenzymes that bound to Inh2 beads in the second stage (Fig. 1B). Conversely, PP1 holoenzymes that did not bind Inh2 beads in the first stage were captured on TP-CPI-17 beads in the second stage, but not on Inh2 beads (Fig. 1C). The data show that CPI-17 and Inh2 exclusively recognized and bound to different subsets of PP1 holoenzymes.

The binding of myosin phosphatase to TP-CPI-17 and Inh2 was analyzed further by immunoblotting (Fig. 2). MYPT1 was recovered only from TP-CPI-17 (Fig. 2A Top), even though about equal amounts of PP1C holoenzymes bound to TP-CPI-17 and Inh2 (Fig. 2A Middle). As a control, lysates were adsorbed to MC-LR, an active site inhibitor, to bind the PP1C and PP2A phosphatases in the lysate (Fig. 2A). The amount of bound MYPT1:PP1C (myosin phosphatase) relative to the amount of PP1C was compared for both inhibitors (Fig. 2B). In this analysis, MYPT1:PP1C binding to TP-CPI-17 beads was enriched by 9.0-fold compared to Inh2 beads and 2.6-fold com-
We determined that binding of TP-CP1-17 to myosin phosphatase occurs at the active site of PP1C (Fig. 3). COS7 cells were transiently transfected with WT myc-tagged MYPT1 or MYPT1 mutated (F38A) in the putative PP1C-binding site. The cell lysate was split, and one-half was subjected to immunoprecipitation by using anti-myc antibody (Fig. 3A). Both myc-MYPT1(WT) and myc-MYPT1(F38A) were expressed and precipitated effectively, but PP1C was detected only with myc-MYPT1(WT), showing that the F38A mutation eliminated PP1C binding to MYPT1. The other half of the lysate was used in a binding assay with TP-CP1-17 beads (Fig. 3B). The myc-MYPT1(WT)+PP1C holoenzyme bound to TP-CP1-17 beads, whereas the F38A mutant of MYPT1 was not recovered, even though endogenous PP1C bound to these beads. We concluded that MYPT1 did not bind directly to TP-CP1-17 in these assays, but the interaction is indirect and bridged via PP1C. Sites on PP1C for interaction of MYPT1 and TP-CP1-17 are functionally separate. We tested whether the active site inhibitor MC-LR would compete with TP-CP1-17 binding to myosin phosphatase (Fig. 3C). Binding assays were performed by using MC-LR, TP-CP1-17 or GST-MYPT1(1–300) as ligands, in the presence or absence of excess MC-LR in cell lysates. In the control, addition of MC-LR fully eliminated PP1C binding to MC-LR beads. Likewise, addition of MC-LR prevented PP1C binding to TP-CP1-17 beads. However, MC-LR added to the lysate did not reduce binding of PP1C to GST-MYPT1(1–300). Binding to TP-CP1-17 requires the unoccupied active site of PP1C, but MYPT1 can bind PP1C with MC-LR at the active site.

**Specific Inhibition of Myosin Phosphatase by Differential Dephosphorylation of CP1-17.** Consistent with its preferential binding, CP1-17 is a highly specific inhibitor of myosin phosphatase (Fig. 4). Myosin phosphatase prepared from porcine aorta smooth muscle consisted of the PP1C δ isoform and a fragment of MYPT1 (9). Both TP-CP1-17 and P-CP1-17 inhibited myosin phosphatase with the same potency (IC50 = 3 nM), as described (9, 22,
and muscle? Osin phosphatase binds P-CPI-17 but hydrolyzes it so slowly that dephosphorylates P-CPI-17 to alleviate inhibition, whereas myosin inhibit the activity. These results show glycogen-bound PP1 phospho-PHI-1, an analog of CPI-17 (28), could completely phosphatase was exclusively PP1, because both TP-CPI-17 and the dephosphorylation of the P-CPI-17. The glycogen-bound PP1C isoform (Fig. 4), consistent with the report by Colbran et al. (31). Surprisingly, P-CPI-17 was a relatively poor inhibitor of this PP1C, and it was inhibited by TP-CPI-17 with an IC50 of 100 nM, 30-fold higher compared with myosin phosphatase (Fig. 4A and B). The glycogen-bound PP1C mainly consists of PP1δ isoform (Fig. 4C), consistent with the report by Colbran et al. (31). Surprisingly, P-CPI-17 was a relatively poor inhibitor of this glycogen-bound PP1, with a potency the same as the unphosphorylated inactive CPI-17 (Fig. 4B). This raised the possibility that CPI-17 was dephosphorylated in the assay. Indeed, Thr-38 of P-CPI-17 was dephosphorylated during the assay with the glycogen-bound PP1 (Fig. 4D). In contrast, P-CPI-17 remained phosphorylated in the parallel assay with myosin phosphatase (Fig. 4D). Because these assays were carried out in the presence of 1 nM okadaic acid and 0.1 mM EDTA, any traces of PP2A, PP2B, or PP2C activities were unlikely to be responsible for the dephosphorylation of the P-CPI-17. The glycogen-bound phosphatase was exclusively PP1, because both TP-CPI-17 and phospho-PHI-1, an analog of CPI-17 (28), could completely inhibit the activity. These results show glycogen-bound PP1C dephosphorylates P-CPI-17 to alleviate inhibition, whereas myosin phosphatase binds P-CPI-17 but hydrolyzes it so slowly that an inactive complex accumulates.

Is PP1 responsible for dephosphorylation of CPI-17 in smooth muscle? α-Toxin-permeabilized rabbit femoral artery was treated with various phosphatase inhibitor compounds, and phosphorylation of Thr-38 in endogenous CPI-17 was assayed (Fig. 5A). Addition of calyculin A (1 μM) increased CPI-17 phosphorylation 7-fold compared with basal levels. On the other hand, okadaic acid (1 μM) or fostriecin (3 μM), both inhibitors more selective for PP2A relative to PP1, did not increase phosphorylation of CPI-17. Inhibition of PP2A by fostriecin was verified by the appearance of increased P-Thr in 90- and 20-kDa proteins by phospho-specific immunoblotting (Fig. 5A Inset). The results indicate that Thr-38 in P-CPI-17 is dephosphorylated by PP1 in smooth muscles.

**Discussion**

In this study, we provide evidence that phosphorylated CPI-17 binds and inhibits myosin phosphatase and is selective for myosin phosphatase among various PP1 holoenzymes in cells. Immunoblots with an isomeric-specific antibody of PP1C showed that both myosin phosphatase and glycogen-bound phosphatase contain the same δ isoform of PP1 (Fig. 4C) (31), so the selectivity of P-CPI-17 cannot be ascribed to sequence differences in the catalytic subunit. Instead, we propose that the inhibitor is a phosphoprotein, and P-CPI-17 may contribute to PP1C produced by interactions with different regulatory subunits. Regulatory subunit association with PP1C is known to
exert allosteric control on substrate specificity (5), and here we show binding to different PP1 inhibitor proteins is affected by PP1 regulatory subunits. Affinity chromatography (22) and the sequential pull-down assay done here separate PP1 holoenzymes in cell lysates into at least two groups, those that bind or do not bind to CPI-17. Specific binding of CPI-17 to myosin phosphatase was eliminated by addition of MC-LR, indicating CPI-17 docks primarily at the active site of PP1C associated with MYPT1. Alternatively, interaction of G\textsubscript{M} with PP1C affects the conformation at the active site to reduce the relative affinity for CPI-17 and also allow PP1C to dephosphorylate CPI-17. Thus, glycogen-bound PP1 evades inhibition by CPI-17 by inactivating the inhibitor, by dephosphorylation. The PP1 holoenzymes that dephosphorylate CPI-17 appear insensitive to inhibition, whereas PP1I holoenzymes (i.e., myosin phosphatase) that poorly dephosphorylate CPI-17 are thereby sensitive to inhibition. Previous studies showed that the dephosphorylation rate of P-CPI-17 by myosin phosphatase is <0.1 sec\textsuperscript{-1}, which is 50-fold slower compared with P-LEC20 substrate (30). Furthermore, P-CPI-17 and TP-CPI-17 were equally potent toward myosin phosphatase, consistent with no dephosphorylation during assay (Fig. 4 A and D). Therefore, P-CPI-17 binds myosin phosphatase and forms a transiently inactive complex. We propose that the inhibitory specificity of CPI-17 is determined by relative rates of dephosphorylation by different PP1 holoenzymes (Fig. 5B).

This model has the advantage that it does not require other phosphatases to dephosphorylate and inactivate CPI-17. Thiophosphorylated CPI-17 captured essentially no PP2A from cell lysates, indicative of relatively weak binding. Even though there are reports that PP2A can dephosphorylate purified CPI-17 in biochemical assays, we think it unlikely that PP2A dephosphorylates CPI-17 in vivo. Supporting this view, inhibition of PP1, but not PP2A, -2B, or -2C, enhanced phosphorylation of CPI-17 in permeabilized artery strips (Fig. 5A). Another consideration is the high expression level of CPI-17 in arteries, where the concentration is estimated at 7 nM (32). Upon activation (phosphorylation of Thr-38) of CPI-17, one would predict that all PP1 in the cell would be inhibited, because, as an example, CPI-17 has an IC\textsubscript{50} of 100 nM for glycogen-bound PP1. A disaster is averted by efficient dephosphorylation of CPI-17 by different PP1I holoenzymes. This concept is critical to account for restricted inhibition of a select pool of PP1I holoenzymes. Myosin phosphatase containing the MYPT1 subunit is one such pool of CPI-17-sensitive phosphatase. However, this does not imply that MYPT1-containing phosphatase is the only CPI-17-sensitive holoenzyme. Related regulatory subunits such as p85 (33), or other subunits, may also produce the conformation of PP1C that binds phospho-CPI-17 with high affinity and restricted catalytic activity. These would be CPI-17-sensitive by the same proposed mechanism.

P-CPI-17 inhibits native PP1C monomer purified from rabbit muscle with IC\textsubscript{50} of 10 nM (9). The IC\textsubscript{50} value is 5-fold lower against myosin phosphatase and 10-fold higher against glycogen-bound PP1. This implies that binding of MYPT1 alters the conformation of PP1C to increase sensitivity to CPI-17, whereas interaction with other targeting subunits, such as G\textsubscript{M}, reduces affinity for CPI-17 and facilitates hydrolysis of P-CPI-17. The interaction of PP1 and CPI-17 is sensitive to the inhibitor conformation as well, because the Y41A mutant of CPI-17 is dephosphorylated by myosin phosphatase as efficiently as the preferred substrate phospho-myosin light chain (30). The NMR solution structure of CPI-17 reveals an N-terminal loop consisting of the phosphorylation site, called the P-loop, followed by a four-helix bundle (34, 35). The Asp mutation at Thr-38, which mimics phosphorylation, exposes the P-loop on the molecular surface (35). In the T38D mutant and phosphoT38 structure, the side chain of Tyr-41 tethers P-loop to B-helix of CPI-17, suggesting that Tyr-41 could stabilize P-loop structure to prevent hydrolysis of P-CPI-17 at the active site of myosin phosphatase.

Purified myosin phosphatase is also inhibited by phosphorylated forms of PHI-1 and KEPI, members of a family of inhibitors related to CPI-17 (28, 36, 37). Amino acid sequences are highly conserved within the domain required for potent inhibition of PP1I holoenzymes (called the phosphatase holoenzyme inhibitory domain) that includes the Tyr-41 residue in the P-loop. Therefore PHI-1 and KEPI are also likely to interact with the PP1C active site. We propose that the mechanism described here might apply in parallel to these other PP1 phosphoinhibitor proteins. Interestingly, unlike CPI-17, P-PHI-1 inhibits glycogen-bound PP1 with an IC\textsubscript{50} of 30 nM (9). Thus, differences in the conformation of PHI-1 relative to CPI-17 may protect it from dephosphorylation by glycogen-bound PP1. Conformations of PP1C induced by regulatory subunit association would allow either (i) binding and dephosphorylation or (ii) binding and inhibition.

Because both phospho-I-1 and -Inh2 were unable to inhibit glycogen-bound PP1, since that time it has been accepted that the action of these inhibitors required dissociation of PP1I holoenzymes to release the monomeric catalytic subunit (38). The KIOF sequence of I-1 and IKGI sequence of Inh2 are thought to function as the canonical VXF motif for PP1C binding. This concept makes interaction of PP1C with inhibitor proteins and targeting subunits mutually exclusive (4, 39). Nevertheless, there are now several lines of evidence that indicate direct interaction of I-1 and Inh2 with PP1I holoenzymes, without dissociation (11–13). These results lead us to propose a model for specific regulation of PP1I holoenzymes by inhibitor proteins, where the inhibitor has narrow specificity for a pool of PP1I holoenzymes. All of the inhibitor proteins can inhibit PP1C monomer. However, interaction with regulatory subunits occludes some sites and allosterically modifies the available active site to restrict PP1C binding to particular inhibitor proteins. Indeed, I-1 and Inh2, as well as CPI-17, each exhibits activity against a select pool of PP1I holoenzymes. It remains unclear how, or even whether, the motifs that resemble VXF in I-1, Inh2, PHI-1, and KEPI interact with PP1 in holoenzymes. In fact, most structure/function analysis of inhibitor proteins to date has been done using isolated PP1C or Mn\textsuperscript{2+}-activated recombinant PP1C, leaving regulation of PP1I holoenzymes by inhibitor proteins mostly an unexplored issue. Over 50 PP1 targeting subunits are reported, and there are less than a dozen PP1 inhibitor proteins to date. To complete the circuitry that gives specific signaling to individual pools of PP1, we might expect more inhibitor proteins or means of restricted intracellular distribution. In addition, there is regulation via direct phosphorylation of PP1C and regulatory subunits (2, 3). Last, discrimination of PP1C conformations within the context of different holoenzymes holds out hope that small molecule inhibitors of individual PP1I holoenzymes remain a possibility for therapeutic applications.

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