The functional specificity of type 1 protein phosphatases (PP1) depends on the associated regulatory/targeting and inhibitory subunits. To gain insights into the mechanism of PP1 regulation by inhibitor-2, an ancient and intrinsically disordered regulator, we solved the crystal structure of the complex to 2.5 Å resolution. Our studies show that, when complexed with PP1c, I-2 acquires three regions of order: site 1, residues 12–17, binds adjacent to a region recognized by many PP1 regulators; site 2, residues 130–169, forms α-helical regions that lie across the substrate-binding cleft. Specifically, residues 148–151 interact at the catalytic center, displacing essential metal ions, accounting for both rapid inhibition and slower inactivation of PP1c. Thus, our structure provides novel insights into the mechanism of PP1 inhibition and subsequent reactivation, has broad implications for the physiological regulation of PP1, and highlights common inhibitory interactions among phosphoprotein phosphatase family members.

Phosphorylation, a fundamental mechanism for the regulation of protein function, is mediated by the coordinated action of protein kinases and phosphatases. Protein phosphatase 1 (PP1) is a major Ser/Thr protein phosphatase that controls a myriad of cellular processes such as glycogen metabolism, muscle contraction, cell cycle, gene expression, and neuronal activity. The pleiotropic actions of PP1 are determined by its associated regulatory components that direct the enzyme to various subcellular compartments in the proximity of substrates and/or modulate phosphatase activity (1–3). The human genome contains only three PP1 catalytic subunit (PP1c) genes encoding four isoforms α, β, γ1, and γ2, with the latter two generated by alternative splicing. However, close to 100 PP1c-binding proteins have been reported, the majority of which contain an RXVF, or its variant RXVXT, motif that binds to a hydrophobic surface groove located on a surface behind the PP1c active site (4). The ability of multiple proteins to bind by this mechanism in part accounts for the wide range of functions performed by this phosphatase.

Inhibitor-2 (I-2) was the first protein phosphatase regulator identified (5) and is widely expressed from yeast to man. Mammalian I-2 forms a stable and high affinity ($K_d = \sim 2$ nM) (6) complex with PP1c termed the ATP-Mg$^{2+}$-dependent phosphatase (7). Although the molecular basis for its action has remained unclear, the ability of I-2 to rapidly inhibit PP1 was a critical tool for the identification of PP1 activity in many eukaryotes. I-2 also promotes a slower “inactivation” of PP1c (8–10) to create a latent complex. The reactivation of the latent PP1c-I-2 complex is triggered by phosphorylation of I-2 at Thr$^{72}$ by several protein kinases,7 including GSK-3, ERKs (extracellular signal-regulated kinases), and cyclin-dependent kinases (CDKs) (11–13), but full activity toward other substrates is not elicited until Thr$^{72}$ is dephosphorylated in an autocatalytic manner.

Transgenic expression of I-2 in mice suggests that I-2-mediated inhibition of PP1 regulates cardiac contractility (14). Other studies that localized I-2 at centrosomes have noted dynamic changes in Thr$^{72}$ phosphorylation during mitosis (15), suggesting a role for the PP1-I-2 complex in cell division. Oxidative stress in neuronal cells, which increased I-2 phosphorylation and PP1 activation (16), also hint at a role for the ATP-Mg$^{2+}$-dependent protein phosphatase in controlling neuronal survival. Thus, understanding the molecular basis by which I-2 modulates PP1 function is critical for establishing the physiological and pathophysiological role of the PP1-I-2 complex and

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7 Prior residue numbering for the mammalian forms of inhibitor-2 has started with the first residue after the initiator methionine on the basis of protein sequencing experiments. This manuscript will adopt the HUGO recommendations and begin numbering at the initiator methionine. Mouse I-2 has a one-residue insertion at homologous position 21, consequently residues C-terminal to this insertion will be incremented by two (i.e. Thr$^{74}$ versus Thr$^{72}$).

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assessing the contribution of dynamic control of this protein phosphatase complex in human health and disease.

The purified I-2 protein has a low sedimentation coefficient (1.75 S) and a large Stokes radius (3.5 nm) and is both heat- and acid-stable. These properties are consistent with the mostly random coil structure suggested by solution NMR, which showed only a single area of weak α-helix comprising residues 135–143 (17). I-2 appears to belong to a class of intrinsically disordered proteins that acquire structure when associated with partner molecules (18). Here we report the structure of the rat PP1cγ and mouse I-2 complex to 2.5 Å resolution. Analysis of this structure provides new insights into the mechanism by which I-2 inhibits and inactivates PP1c and highlights common structural determinants that mediate the inhibition of protein serine/threonine phosphatases.

MATERIALS AND METHODS

Expression, Purification, and Activity Measurements—The complex between rat PP1cγ1 and mouse I-2 was produced through coexpression of the two proteins in Escherichia coli BL21 cells using two vectors. The rat His-tagged PP1cγ, in which six histidine codons were introduced at the N terminus, was expressed using the pTacTac vector (19), and the mouse I-2 was expressed using a modified pACYC vector (New England Biolabs) to introduce a T7 promoter. These proteins were produced by induction with isopropyl β-D-thiogalactoside and incubation of the cells overnight at 16 °C. The cells were lysed using a French pressure cell and a clarified supernatant obtained by centrifugation at 100,000 × g. The complex was purified without the addition of supplemental MnCl2 in a two-step procedure where nickel-nitrilotriacetic acid chromatography was directly followed by gel filtration on a Superdex G75 column. The complex was greater than 95% pure as judged by SDS-PAGE.

The in vitro reconstituted complexes were prepared by modification of a previously described procedure (20). Briefly, PP1cγ or PP1co prepared in the present of Mn2+ were incubated with a 2-fold molar excess recombinant rabbit I-2 for 30–40 min at room temperature. The excess I-2 was removed by using a Centricon-50 centrifugal concentrator with repeated centrifugations and redilution until no I-2 could be detected in the effluent. Complex formation and absence of free polypeptides were verified by native PAGE (see Fig. 1a). As the free PP1c did not enter the native gel under the conditions used, analysis of the complexes by SDS-PAGE was performed, which indicated that I-2 and PP1c were present at an apparent equimolar ratio (data not shown). The native PP1c-I-2 complex was purified from rabbit skeletal muscle as previously described (21). Phosphatase activity was measured using glycerogen phosphorylase a as a substrate, where an appropriately diluted phosphatase sample was incubated with 1 mg/ml 32P-labeled phosphorylase a (300–400 cpm/pmol) in 50 mm Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.2% 2-mercaptoethanol, 5 mM caffeine, and 0.2 mg/ml bovine serum albumin for 10 min at 30 °C. The reaction was terminated by trichloroacetic acid precipitation and 32P release monitored by scintillation counting. Activation of the PP1c-I-2 complex was measured by preincubation with GSK-3β in the presence of ATP and magnesium acetate, prior to assaying for phosphatase activity. The reaction conditions were typically 50 mm Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.2 mm ATP, 2.5 mm magnesium acetate, 0.5 μg/ml GSK-3β, and 0.4 μg/ml of the complex for 10 min at 30 °C with or without the presence of 0.2 mm manganese chloride. To measure reactivation by trypsin/Mn2+, the complex was incubated with 1 μg/ml trypsin, ± 0.6 mm MnCl2, for 5 min at 30 °C. Soybean trypsin inhibitor was added to stop the reaction, and phosphatase activity was measured as described above.

Crystallization and Structure Determination—The crystals of the His-PP1cγ1-I-2 complex were grown using sitting drop vapor diffusion from solutions containing 7 mg/ml of the complex. Two separate conditions were found to produce crystals of the complex. The first condition contained 100 mm Tris-HCl, pH 7.5, 300 mm potassium acetate, 12% (w/v) polyethylene glycol 3350, and the second condition contained 100 mm Tris-HCl, pH 8.6, 150 mm sodium citrate, and 20% (w/v) polyethylene glycol 3350. Stable crystals were obtained at 15 °C after ~10 days of incubation. The crystals were flash frozen following the introduction of 25% ethylene glycol in a rapid two-step procedure. All of the diffraction data were collected at beamline 19-ID at the Advanced Photon Source, Argonne National Laboratory. Data reduction and scaling was accomplished using HKL2000 (22). The structure of the complex was solved using the program AMoRe (23) with the structure of human PP1cγ (4) as the search model. Electron density maps produced using this model showed the presence of strong positive difference features at three distinct locations on the surface of each PP1cγ subunit in the asymmetric unit (supplemental Fig. S1). The structure of I-2 was built into the available electron density using Coot (24), and the resulting model was subjected to restrained refinement with tight noncrystallographic symmetry restraints on the main chain atoms and medium restraints on the side chain atoms using Refmac5 (25). All structures show >98% of the residues in the allowed regions of their respective Ramachandran plots. Consistent with other PP1c structures, residues Asp95 and Arg98 in each PP1c subunit are found in the disallowed region. All of the I-2 residues fall within the most favored regions.

RESULTS

Biochemical Properties of Native and Recombinant PP1c-I-2 Complexes—A PP1cγ1-I-2 complex was generated by coexpression of the two polypeptides in E. coli in the absence of added metal ions (26). Prior structural studies of PP1cα (27) and PP1cγ (28) utilized proteins expressed in E. coli in the presence of Mn2+, which was required for enzyme activity. Thus, we compared the biochemical properties of the purified coexpressed complex and a complex reconstituted in vitro by incubation of recombinant rabbit I-2 and rat PP1cγ1 expressed in bacteria in the presence of Mn2+ with those of the native PP1c-I-2 complex isolated from rabbit skeletal muscle (21) (Fig. 1, a and b). Whereas the native enzyme was inactive until reactivated following incubation with GSK-3 and ATP-Mg2+, the in vitro reconstituted PP1c-I-2 complex displayed readily measurable phosphorylase phosphatase activity that was not further increased following the addition of GSK-3 and ATP-Mg2+ (Fig. 1b). By contrast, the coexpressed PP1c-I-2 complex, like the native complex, was inactive and could be activated to some
Extent by GSK-3 and ATP-Mg²⁺. Full activity of these complexes was observed only when Mn²⁺ was also included in the activation reaction. Digestion with trypsin in the presence of Mn²⁺ has been widely used to degrade regulatory subunits and reveal the full activity of the trypsin-resistant PP1 catalytic subunit (29). Indeed, all three PP1-I-2 complexes were fully reactivated by treatment with trypsin in the presence of Mn²⁺. However, only the reconstituted PP1-I-2 complex was significantly activated by trypsin alone (Fig. 1c), suggesting that PP1c was not fully inactivated in the reconstituted complex. Most importantly, despite the absence of added metal ions in the culture media, the PP1c from the coexpressed complex could be fully activated with a specific activity very similar to the native enzyme (Fig. 1, b and c) and isolated recombinant PP1c produced in the presence of Mn²⁺ (Fig. 1d). As anticipated, PP1c expressed in the absence of Mn²⁺ was inactive, even when metal ions were included in the phosphatase assay (Fig. 1d). These data suggested that the coexpressed PP1-I-2 complex more closely resembled the native ATP-Mg²⁺-dependent phosphatase complex.

Structure of the PP1-I-2 Complex: PP1c Binding by I-2—Analysis of free I-2 by CD spectroscopy and through computational approaches (supplemental data and Figs. S2 and S3) suggests that the protein lacks ordered elements of secondary structure, which is consistent with the available NMR data. In contrast, both CD measurements and computational assessment of the propensity for ordered interactions with partner molecules suggest that I-2 can acquire ordered structure when in complex with PP1c (supplemental Figs. S2 and S3). We solved two crystal structures of the stable complex formed between rat PP1c and mouse I-2 to 2.6 and 2.5 Å resolution, respectively (Table 1). Each crystal form contains two independent complexes in the asymmetric unit, and all of the complexes displayed the same mode of interaction between I-2 and PP1c within the resolution of the data. Crystals of the PP1-I-2 complex were dissolved and analyzed by SDS-PAGE and established that both proteins were present (data not shown).

The structure revealed three major regions of interaction between I-2 and PP1c (Fig. 2), all of which are highly conserved through evolution (Fig. 3 and supplemental Fig. S4) and bury ~4700 Å² of surface area in the complex. The first and shortest region of interaction, site 1, involves residues 12–17 (KGILKN) (Fig. 4, a and b) and represents a mixture of hydrophobic associations and hydrogen bonds. For example, the side chain of I-2 Ile¹⁴ is buried in a hydrophobic pocket formed by residues Leu⁵⁵, Phe¹¹⁹, and Leu⁵⁹ in PP1c, whereas the side chain of Leu⁵⁵ of I-2 is buried in a pocket formed by Pro⁶⁰, Leu⁵³, and Phe¹¹⁹ from PP1c. The remain-
ing interactions are contributed by I-2 Lys_{12}, Lys_{16}, and Asn_{17}. In particular, the side chain of I-2 Asn_{17} hydrogen bonds to the main chain carbonyl oxygen and main chain nitrogen of residues 52 and 54, respectively, and I-2 Lys_{16} hydrogen bonds to Asp_{166} in PP1c/H9253.

The second longest stretch of interaction, site 2, occurs between residues 44 and 56 (KSQKWDEMNILAT) of I-2 (Fig. 4c) and a common point of contact between PP1c/H9253 and most PP1-binding proteins, namely the RVXF groove. The sequence of residues in I-2 bound to this site is KSQKW where the Gln and Trp residues fill in the positions occupied by Val and Phe in many PP1 regulators. Similarly to the interactions with the Phe residue present in the peptide derived from RGL (4) and the MYPT1 fragment (30), the aromatic side chain of I-2 Trp_{48} binds within the hydrophobic pocket formed by Leu_{243}, Phe_{257}, Cys_{291}, and Phe_{293} of PP1c/H9253 (Fig. 4d). I-2 Gln_{46} is located in the same pocket formed by Ile_{169}, Leu_{243}, Leu_{289}, and Cys_{291} of PP1c/H9253, as is the Val residue present in the R_{GL} and MYPT1 complexes. However, the most significant and unique interaction between I-2 and PP1c/H9253 at this site is the side chain amide nitrogen of I-2 Gln_{46} that forms a hydrogen bond with the sulfhydryl group of PP1c/H9253 Cys_{291} (Fig. 4d). In addition, the side chain of the first residue in this stretch, I-2 Lys_{44}, interacts with PP1c/H9253 Asp_{166}, the same residue to which I-2 Lys_{16} forms a hydrogen bond.

The Structure of the PP1-I-2 Complex: Mode of PP1c Inhibition by I-2—The longest contiguous stretch of interaction occurs between residues 130 and 169 of I-2 and the active site of PP1c/H9253. This stretch of I-2 adopts an extended $\alpha$-helical structure that is disrupted by five residues between amino acids 149 and 153. Residues 130–146 lie along the “acidic groove,” to position amino acids 147–151 in the active site of PP1c,
whereas residues 152–169 exit the active site to the adjacent proposed "hydrophobic substrate-binding groove" of PP1c and results in the displacement of one or more of the metal ions located in the active site. Prior structures of PP1c and PP1c showed a bimetallic metal center, where M1 and M2 were both Mn(II) in PP1c (27), whereas the PP1c structure contained one Fe(II) and one Mn(II) (28). A key difference between these structures and that of our PP1c-1 complex is that the metal ion is missing from the M1 site in both of our crystal structures. The complex crystallized in the presence of acetate is missing both metal ions. In all of our structures, I-2 Tyr149 forms a hydrogen bond with an ordered water molecule that is positioned near the M1 metal site (Fig. 5c). This water is held in place through additional hydrogen bonds with the catalytic metal ligands, PP1c His66 and Asp64. The side chain of I-2 Asn150 forms a hydrogen bond with PP1c Tyr134, which lies adjacent to Arg96 in the active site. Lastly, I-2 Glu151 hydrogen bonds to PP1c Arg96 and His125 and interacts with PP1c Arg221 through a water molecule. In summary, the interactions of residues 148–151 from I-2 within the catalytic site of PP1c most likely mediate the inhibitory action.

**DISCUSSION**

**Importance of the PP1-I-2 Structure**—The three-dimensional structure of PP1c was first resolved over a decade ago and highlighted key features of a bimetallic catalytic center and an overall pattern of protein folding conserved in other protein serine/threonine phosphatases (31–35). Subsequent studies co-crystallized PP1c with a synthetic peptide derived from R_gly (4), a glycogen targeting subunit. These studies identified a site some distance from the catalytic center, where sequences homologous to RVXF found in most PP1 regulators interact and facilitated the identification of additional PP1 regulators. More recently, PP1c was co-crystallized with a fragment of MYPT1, a PP1 regulator found in smooth muscle (30). This structure confirmed the commonality of interac-

### Table 1

Data collection and refinement statistics

| Potassium acetate | Sodium citrate |
|-------------------|----------------|
| **PP1c-I-2 complex** |
| Space group       | a = 95.4, b = 103.5, c = 151.6 |
| Cell dimensions (Å)| a = 96.3, b = 103.2, c = 150.0 |
| Resolution (Å)    | 43.261 (2.69-2.61) |
| Uniqueness        | 44,466 |
| Redundancy        | 53.5 (5.2) |
| Completeness (%)  | 95.8 (86.0) |
| <i>d</i>/<i>d'</i> | 15.5 (2.7) |
| R<sub>merge</sub>  | 0.085 (0.56) |
| Refinement        | 0.090 (0.61) |
| No. reflections   | 50.250 (2.59-2.50) |
| R<sub>merge</sub>/R<sub>free</sub> | 0.194/0.253 (0.322/0.333) |
| No. protein atoms | 5743 |
| No. solvent atoms | 99 |
| Average B-factor (PP1c) | 44.0 Å<sup>2</sup> |
| Average B-factor (I-2 only) | 61.5 Å<sup>2</sup> |
| Coordinate error (R<sub>merge</sub>) | 0.26 Å |
| Root mean square deviation bond lengths | 0.010 Å |
| Root mean square deviation bond angles | 1.19° |

*Parentheses indicate the data statistics for the highest resolution shell used for each structure.
tions along the RVXF motif groove and highlighted interactions along the C terminus of PP1c that imparts isoform specificity to some PP1c interacting proteins. The PP1c−I-2 complex further extends our understanding of protein phosphatase regulation and demonstrate that with “inhibitor proteins” like I-2, which lacked significant ordered structure, the association with PP1c induces conformations that specifically enhances association with PP1c. I-2 appears not to modify the overall structure of PP1c(4, 28), but the direct association with the PP1 catalytic center and its ability to displace critical catalytic metals provides a clear molecular basis for understanding its ability to inhibit and slowly inactivate the protein phosphatase. It is noteworthy that I-2 is among the most ancient of PP1 regulators and is evolutionary conserved primarily in the observed regions of interaction.

Association of I-2 with PP1c—Numerous studies have exploited site-directed mutagenesis of both PP1c and I-2 to gain a better understanding of the mode of I-2 action. These studies highlighted regions in I-2 whose deletion or mutation either abrogated PP1c binding or attenuated I-2 activity (20, 36–39). The structure of the complex confirms that previously identified regions correspond to domains in our structure that convey critical interactions with PP1c and for the first time suggest the potential mechanism by which I-2 inhibits PP1 activity.

The interactions at site 1 involve residues 12–17 that bind primarily to a surface created by amino acids 50–59 in the

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**FIGURE 4. Interactions of residues 12–17 and 44–56 from I-2 with PP1c.**

(a) a GRASP (56) electrostatic surface representation of PP1c shows how residues 12–17 interact with the surface characteristics of PP1c. The positions of the four acidic residues on the surface of PP1c that surround Lys16 of I-2 are labeled. b, stereo view the interactions contributed by residues 12–17 of inhibitor with PP1c. Atom-type coloring is used, with gray representing carbon atoms in the structure of PP1c and yellow representing carbon atoms in I-2 atoms. Selected residues in PP1c are labeled using the three-letter amino acid code, and residues in I-2 are labeled using the single-letter amino acid code. c, region of PP1c to which residues 44–56 from I-2 are bound. The molecular surface PP1c is represented in gray, and selected residues in PP1c are labeled using the three-letter amino acid code. The structure of I-2 is represented using atom-type coloring and labeling as described for b, d, stereo view the interactions contributed by residues 46–50, 52, and 53 of I-2 with PP1c. Atom-type coloring and labeling are as described for b.
PP1c\textsubscript{y} subunit. We had previously shown that deletion of the N-terminal 35 residues of I-2 abrogated inhibitory potency (20, 37). An interaction between residues 10 and 14 in I-2 and amino acids 54 and 56, as well as residues 166–168 of PP1 was later predicted by complementary mutagenesis of PP1 and I-2. Mutation of I-2 Lys\textsubscript{12} and Ile\textsubscript{14} resulted in over 500-fold reduction in the inhibitory potency of I-2 for PP1 (38), whereas mutation of Glu\textsubscript{54} and Glu\textsubscript{56} in PP1c resulted in a 10-fold decrease in its sensitivity to inhibition by I-2 (40). The most telling set of mutations included PP1c Asp\textsubscript{166}, which when combined with those of Glu\textsubscript{167} and Lys\textsubscript{168}, essentially abolished PP1 inhibition by I-2, decreasing its inhibitory potency by over 800-fold (40). Thus, hydrogen bonding between PP1c Asp\textsubscript{166} and I-2 Lys\textsubscript{14}, as well as I-2 Lys\textsubscript{44}, is a major interaction and contributes up to 3.9 kcal/mol of binding energy. Interestingly, the side chain of I-2 Lys\textsubscript{16} lies along the surface of PP1c and runs between PP1c Glu\textsubscript{54} and Glu\textsubscript{56} to interact with PP1c Asp\textsubscript{166} (Fig. 4b). We would suggest that the primary role of PP1c Glu\textsubscript{54} and Glu\textsubscript{56} is to maintain a negatively charged surface that attracts and orients I-2 Lys\textsubscript{12} and Lys\textsubscript{14} properly to promote the interactions contributed by I-2 Ile\textsubscript{14} and Leu\textsubscript{15} and the side chain of Asn\textsubscript{17}. Although some mutagenesis work pointed to I-2 Ile\textsubscript{11} as an important contributor to I-2 activity (38), characterization of the Caenorhabditis elegans and Drosophila I-2 proteins suggest that the residue corresponding to Ile\textsubscript{11} is not critical in these species, but the more highly conserved segment including residues 12–17 represents a common area of interaction with PP1 (41). Consistent with this notion, forms of I-2 lacking this region, such as rat I-2\textsubscript{B} (42), or lacking sequence N-terminal to this site, such as PP1c Glc\textsubscript{8} (Fig. 3) and Drosophila I-2 isoform (I-1) (43), are significantly weaker PP1 inhibitors.

The interactions at site 2 involve a region of I-2 comprised of residues 44–56. As mentioned above, Asp\textsuperscript{166} in PP1c\textsubscript{y} mediates one of the contacts to residues 44–56 that binds within the RVXF binding groove that is occupied by many PP1 regulators (4). The specific sequence in I-2 that docks in this groove is KSQKW where the Gln and Trp occupied the positions of Val and Phe found in other PP1 regulators (Figs. 4d and 6a). Support that this site is involved in I-2 interaction with PP1c comes from previous mutagenesis studies, where the Trp was replaced by Ala, showing that the mutation I-2 exhibited a 10-fold increase in IC\textsubscript{50} (20). Similar results were also reported by mutation of the corresponding Phe in the Drosophila ortholog (39). Recent computational studies derived a consensus sequence for PP1c binding as [HKR][ACHKNQRSTV]\textsubscript{2}[V][CHKNQRST][FW] (44), which does not predict the KSQKW sequence in I-2 as a PP1c-binding region. In this regard, it is worth noting the all mammalian I-2 proteins contain a Gln in this position, whereas the D. melanogaster and C. elegans orthologs contain Ala (Fig. 3). The latter substitution is even more remarkable when one considers that substitution of Val to Ala is a commonly used strategy for abrogating PP1 binding by many PP1 regulators. Despite the apparent dissimilarity between Gln and Val, the aliphatic C\textsubscript{\alpha} and C\textsubscript{\beta} atoms of I-2 Gln\textsubscript{56} interact with PP1c similarly to Val, and most importantly, the side chain amide nitrogen of I-2 Gln\textsubscript{56} hydrogen bonds with the sulfhydryl of PP1c Cys\textsuperscript{294} (Fig. 4d), which is highly conserved in all PP1c isoforms (45) and may contribute to the ability of I-2 to potently inhibit all PP1 catalytic subunits. In contrast, the equivalent position in PP2A is Tyr\textsuperscript{284}, and this substitution as well as others in and around the corresponding binding site for residues 12–17 of I-2 accounts for the inability of I-2 to inhibit PP2A activity, despite the highly conserved active site structures in the PP1 and PP2A phosphatases (31, 32).

Emerging studies show that substitution of the Phe within the RVXF motif with many other residues has even more profound effect on PP1c binding such that the single amino acid substitution of Phe to Ala in a very diverse set of PP1 regulators results in a reduction of PP1c binding and regulation by several orders of magnitude. Although the D. melanogaster and C. elegans I-2 orthologs retain Phe in this location, all other I-2 proteins possessed Trp. Phage display studies had hinted that bulky hydrophobic groups, such as Trp and Tyr may also be accommodated in place of Phe and allow PP1c binding (46).

This resulted in prior discussion that another I-2 sequence, KLHY, may dock in this location (20). This work demonstrates that although KLHY does indeed lie within a PP1 interaction...
Overall, the similarities among the RVXF groove interactions in the I-2, P_{GL} peptide, and MYPT1 structures are remarkable. The aligned peptides exhibit a root mean square deviation of 0.8 Å for all main chain atoms within the five residue stretch that includes one residue on either side of I-2 Gln16 and Trp18 (Fig. 6a). The similarity between MYPT1 and I-2 is significantly less outside this stretch because helical segments from each protein pursue very different paths along the PP1c surface (Fig. 6b). The alignment of the MYPT1 and I-2 complexes suggest that considerable steric interference would occur if a heterotrimeric complex were attempted, explaining prior observations that the myosin phosphatase was relatively resistant to inhibition by I-2. 

Absence from PP1c in the PP1-I-2 complex. An ordered water molecule resides near the M1 metal site and I-2 Tyr149 interacts directly with this water molecule, which is held tightly in position by hydrogen bonds to the ligands of the displaced metal ion, PP1c His143 and Asp64. To further anchor this stretch of residues in the active site of PP1c, I-2 Glu151 hydrogen bonds to PP1c Arg216, His223, and Arg221 through another water molecule. Like Arg216, Arg221 also helps bind and position the phosphoryl group during catalysis, and His223 is the proton donor for the reaction. Thus, the I-2 sequence HYNE may be directly responsible for both the inhibition, through occluding the catalytic site, and inactivation of PP1 by preventing binding or promoting displacement of catalytic metals. In support of this latter hypothesis, the addition of Mn^{2+} ions during expression and purification did not lead to the incorporation of metals into the PP1c-I-2 complex (structure not shown). We propose that

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**Common and unique interactions across the surface of PP1c.** a, structure alignment of residues from R_{GL} (green), MYPT1 (blue), and I-2 (red) that bind along the RVXF binding groove. The identity of any residues at each position is indicated using the single-amino acid code in the appropriate color. b, structure alignment of the PP1c-MYPT1 (Protein Data Bank code 1wao, blue and red, respectively) complex to the PP1c-I-2 complex (cyan and magenta, respectively).

**Inhibitory interactions within the active sites of phosphoprotein phosphatase family members.** a, structural alignment of the PP1c-I-2 complex to the structure of calcineurin with its autoinhibitory domain bound in its active site (Protein Data Bank code 1au1). Active site residues in both structures are colored using CPK colors where light gray indicated carbon atoms, blue is nitrogen, and red is oxygen. The positions of the bound zinc and iron ions in the calcineurin structure are indicated as green spheres, and their associated water molecules are blue spheres. The positions of the bound manganese ion and the associated active site waters in the PP1c-I-2 complex structure are indicated as gray and cyan spheres, respectively. The relative positions of the inhibitory Glu151 (E481) from the calcineurin structure (yellow) and of Tyr149 (Y149) and Glu151 (E151) from I-2 (magenta) are indicated. b, structural alignment of the PP1c-I-2 complex to the structure of PPS with the autoinhibitory E76 (yellow) bound in its active site (Protein Data Bank code 1wao). Active site residues in both structures and residues contributed by I-2 are colored as indicated in a. The positions of the bound manganese ions in the PPS structure are indicated as green spheres. The position of the bound manganese ion and the associated active site waters in the PP1c-I-2 complex structure are indicated as gray and cyan spheres.
Structure of the PP1-Inhibitor-2 Complex

I-2 Tyr^{149} promotes the displacement of the catalytic metal(s) in a time-dependent manner, which would explain the slow transition from inhibition to inactivation of the complex.

The region containing Thr^{74} is not visible in this nonphosphorylated PP1c-I-2 complex, making it difficult to accurately predict the role of phosphorylation in reactivating the PP1c-I-2 complex. We speculate that phosphorylation induces yet another conformation of I-2 that promotes displacement of the HYNE sequence and reloading of metals into the active site to catalyze the autodephosphorylation of the threonine. Subsequent structural rearrangements could allow access of substrates to this activated phosphatase complex. Indeed, activation of both the recombinant and the native PP1c-I-2 complexes requires Mn^{2+} in addition to trypsin or GSK-3, indicating that metal ions may not be present in the native complex. Clearly, co-crystallization of PP1c with phosphorylated I-2 will be needed to fully delineate the mechanism of kinase-mediated reactivation of the latent phosphatase complex, but the extensive conservation of the sequence surrounding Thr^{74} in all known I-2 proteins lends support to this hypothesis (Fig. 3).

Relevance of PP1-I-2 Structure for Understanding Physiological Regulation of Other Protein Serine/Threonine Phosphatases—It is noteworthy that the mode of PP1c inhibition by I-2, specifically the region encompassed by residues 130–169, is reminiscent of the binding of the autoinhibitory domain near the catalytic site of calcineurin (52). The autoinhibitory domain of calcineurin does not displace the catalytic metals, instead the side chain of Glu^{481} from calcineurin interacts indirectly with the metals by forming hydrogen bonds with their bound water molecules. Similarly, Glu^{76} within the autoinhibitory domain of PP5 interacts with Tyr^{461} (equivalent to Tyr^{277} in PP1cγ) and may indirectly interact with the active site metals (34). Structural alignment of calcineurin and PP5 with our PP1cγ-I-2 complex shows that Glu^{481} from calcineurin superimposes onto Tyr^{149} from I-2 and Glu^{76} from PP5 is situated between Tyr^{149} and His^{148} in I-2 (Fig. 7). The major difference between these inhibitory interactions in the three phosphatases is the much deeper penetration of Tyr^{149} in I-2 within the PP1c active site, which could promote the displacement of metal ions. It is an attractive speculation that I-2 and possibly other phosphatase inhibitors may have evolved similar strategies for phosphatase regulation.

In summary, the free form of I-2 is largely disordered, but it acquires three regions of ordered structure upon interaction with PP1c. The structural organization is directly responsible for recruitment of I-2 and inhibition of PP1c. Based on these and other studies, initial interactions of I-2 with PP1c may occur via site 1 and site 2, which position residues 130–169 at or near the catalytic site and occupies a significant portion of the substrate-binding site. Subsequent displacement of one or both metals results in inactivation of the phosphatase complex, which then requires the phosphorylation of I-2 and the accompanying rearrangement to allow reinsertion of metals and reactivation of the enzyme. Although some aspects of this regulatory cycle await further structural evidence, the resolution of the PP1-I-2 structure provides first insights into the mode of PP1 regulation by I-2 and possibly other endogenous protein inhibitors.

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