Characterization of the Inhibition of Protein Phosphatase-1 by DARPP-32 and Inhibitor-2*

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Hsien-bin Huang‡‡, Atsuko Horiuchi‡, Takuo Watanabe‡, Su-Ru Shi‡, Huey-Jen Tsay¶, Heng-Chun Li, Paul Greengard∥, and Angus C. Nairn‡‡‡

From the ‡Institute of Biochemistry, Tzu Chi College of Medicine and Humanities, Hualien 970, Taiwan, the ¶Laboratory of Molecular and Cellular Neuroscience, the Rockefeller University, New York, New York 10021, the ∥Institute of Neuroscience, National Yang-Ming University, Taipei 112, Taiwan, and the ‡Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029

Phospho-DARPP-32 (where DARPP-32 is dopamine- and cAMP-regulated phosphoprotein, Mω 32,000), its homolog, phospho-inhibitor-1, and inhibitor-2 are potent inhibitors (IC50 ~1 nM) of the catalytic subunit of protein phosphatase-1 (PP1). Our previous studies have indicated that a region encompassing residues 6–11 (RKKIQF) and phospho-Thr-34, of phospho-DARPP-32, interacts with PP1. However, little is known about specific regions of inhibitor-2 that interact with PP1. We have now characterized in detail the interaction of phospho-DARPP-32 and inhibitor-2 with PP1. Mutagenesis studies indicate that within DARPP-32 Phe-11 and Ile-9 play critical roles, with Lys-7 playing a lesser role in inhibition of PP1. Pro-33 and Pro-35 are also important, as is the number of amino acids between residues 7 and 11 and phospho-Thr-34. For inhibitor-2, deletion of amino acids 1–8 (I2-(9–204)) or 100–204 (I2-(1–99)) had little effect on the ability of the mutant proteins to inhibit PP1. Further deletion of residues 9–13 (I2-(14–204)) resulted in a large decrease in inhibitory potency (IC50 ~800 nM), whereas further COOH-terminal deletion (I2-(1–84)) caused a moderate decrease in inhibitory potency (IC50 ~10 nM). Within residues 9–13 (PIKGI), mutagenesis indicated that Ile-10, Lys-11, and Ile-13 play critical roles. The peptide I2-(6–20) antagonized the inhibition of PP-1 by inhibitor-2 but had no effect on inhibition by phospho-DARPP-32. In contrast, the peptide D32-(6–38) antagonized the inhibition of PP1 by phospho-DARPP-32, inhibitor-2, and I2-(1–120) but not I2-(85–204). These results indicate that distinct amino acid motifs contained within the NH2 termini of phospho-DARPP-32 (KKIQF, where italics indicate important residues) and inhibitor-2 (IKGI) are critical for inhibition of PP1. Moreover, residues 14–84 of inhibitor-2 and residues 6–38 of phospho-DARPP-32 share elements that are important for interaction with PP1.

Protein phosphatase-1 (PP1) is a major eukaryotic protein that regulates diverse cellular processes such as cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription, and neuronal signaling (1–4). The catalytic subunit of PP1 is regulated by the heat-stable protein inhibitors, inhibitor-1, its homolog DARPP-32 (dopamine- and cAMP-regulated phosphoprotein, Mω 32,000), and inhibitor-2 (1, 2). Phosphorylation of inhibitor-1 at Thr-35 or of DARPP-32 at Thr-34 by cAMP-dependent protein kinase converts either protein into a potent inhibitor of PP1. In contrast, unphosphorylated inhibitor-2 interacts with the catalytic subunit of PP1 leading first to inhibition of enzyme activity and subsequently to an inactive complex, termed Mg-ATP-dependent PP1 (1, 5). The Mg-ATP-dependent form of PP1 can then be re-activated following phosphorylation of Thr-72 of inhibitor-2 by glycosyn synthase kinase-3 (GSK-3).

PP1 is also regulated by its interaction with a variety of protein subunits that act in a manner distinct from the inhibitor proteins and that appear to target the catalytic subunit to specific subcellular compartments (1, 2, 6, 7). These regulatory subunits include the following: the glycogen-targeting proteins, Gm and Gl (8); the myofibrillar-targeting protein, M110 (8); and the nuclear-targeting protein, PNU4TS (9, 10). Our recent studies of DARPP-32 (11), studies of Gm (12), M110 (12, 13), inhibitor-1 (14), and peptide display library analysis (15) have indicated that PP1 interacts with phospho-DARPP-32 and the various binding subunits via a short amino acid motif. The exact sequence of the motif is not identical, but one or more basic amino acids is followed by two hydrophobic residues separated by a variable amino acid. In DARPP-32 the motif is found between residues 6 and 11 (RKKIQF). Furthermore, our studies of DARPP-32 have suggested that residues 6–11 bind to PP1 at a site removed from the active site and that this interaction is not directly involved in inhibition of enzyme activity (11). Inhibition of PP1 by phospho-DARPP-32 requires phospho-Thr-34, which is likely to occupy the active site of the enzyme in a manner in which catalysis cannot take place (11, 16, 17).

The identification of the basic/hydrophobic motif in DARPP-32 and the other binding subunits provides a structural basis for their interaction with PP1 in a mutually exclusive manner. However, much less is known about the interaction of inhibitor-2 with PP1. There is no obvious amino acid sequence identity between inhibitor-2 and DARPP-32 or the other binding subunits. Proteolytic studies failed to identify short regions of inhibitor-2 that retained the properties of the

†† To whom correspondence may be addressed: Rockefeller University, 1230 York Ave., New York, NY 10021. Tel.: 212-327-8871; Fax: 212-327-8788; E-mail: nairn@rockvax.rockefeller.edu.
‡‡ To whom correspondence may be addressed: Tzu Chi College of Medicine and Humanities, 701, Sec. 3, Chung Yan Rd., Hualien, Taiwan. Tel.: 886-38-565-301 (Ext. 7038); Fax: 886-38-578-386; E-mail: huanghb@mail.tcu.edu.tw.
¶¶ The abbreviations used are: PP1, catalytic subunit of protein phosphatase-1; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, Mω 32,000; D32, DARPP-32; I2, inhibitor-2; M110, myofibrillar PP1-binding protein; PAGE, polyacrylamide gel electrophoresis.
holoprotein (18). However, initial truncation mutagenesis studies indicated that residues 1-35 were important for inhibition but not for inactivation and complex formation and that a region of the COOH terminus was required for reactivation (5). Given the lack of information concerning the interaction of inhibitor-2 with PP1, we have carried out a detailed structure-function analysis of the protein. We have also characterized further the roles of amino acids within the PP1 binding motif of DARPP-32 and those surrounding phospho-Thr-34 of the protein. Finally we have used peptide competition studies to compare the interaction of inhibitor-2 and phosho-DARPP-32 with PP1. The results obtained indicate that distinct amino acid motifs contained within the NH2 terminus of phospho-DARPP-32 (KIQIF, where bold indicates important residues) and inhibitor-2 (IKIQF) are critical for inhibition of PP1 and are likely to bind to different sites on the enzyme, both of which are removed from the active site. This study therefore identifies a novel mode of interaction of PP1 with its target proteins and extends our understanding of this growing family of important regulatory molecules that regulate cell signaling through control of serine/threonine dephosphorylation.

EXPERIMENTAL PROCEDURES

Materials—HEPES, EGTA, EDTA, phosphorylase b, phosphorylase kinase, 2-nitro-5-thiocyano benzoic acid, Tris, Brij 35, dithiothreitol, bovine serum albumin, and ATP were obtained from Sigma. [γ-32P]ATP was obtained from NEN Life Science Products. Blue-Sepharose and Sephadryl S-200 were obtained from Amersham Pharmacia Biotech. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described (19). The catalytic subunit of PP1 was purified from rabbit skeletal muscle as described (20). Recombinant Blue-Sepharose and Sephacryl S-200 were obtained from Amersham Pharmacia Biotech. The catalytic subunit of cAMP-dependent protein kinase was purified from bovine heart as described (19). The catalytic subunit of PP1 was purified from rabbit skeletal muscle as described (20). Recombinant PP1 was also prepared from Escherichia coli as described (17). [γ-32P]Phosphorylase a (1–3 × 106 cpm/nmol) was prepared from phosphorylase b by deamidation (21).

Peptide Synthesis—D32-(6–38) (c indicates COOH-terminal cysteine), D32-(4–14), I2-(6–20), I2-(189–204), and M110-(1–40) were prepared by the M. W. Keck Biotechnology Resource Center, Yale University. All peptides were purified by reversed-phase HPLC and had the expected amino acid compositions and mass spectra.

Preparation of Wild-type and Mutant Inhibitor-2—Human inhibitor-2 cDNA (21) was used as a template for truncation and site-directed mutagenesis using polymerase chain reaction. Deletion, insertion, and amino acid substitution mutants were amplified using appropriate primers from 100 ng of DARPP-32 cDNA (all oligonucleotides were synthesized by Operon). The amplified DNA was gel purified, digested with NdeI and BamHI, and subcloned into pET3a (or pET-3cp for TT-tagged inhibitor-2). All mutations were confirmed by DNA sequencing.

Wild-type inhibitor-2 and mutants were purified using a method of the described by Helps et al. (21). BL21 (DE3) (DE3) cells containing the expression plasmids were grown in LB broth with ampicillin (0.1 g/liter) at 37 °C until the absorbance at 600 nm was between 0.6 and 1.0. Isopropyl-1-thio-β-galactopyranoside (final concentration 0.4 mM) was added, and the incubation was continued for 4 h. Bacteria were harvested by centrifugation, and the pellet was resuspended in 100 ml of buffer A (20 mM Tris-HCl (pH 7.5), 0.2 mM N-phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 4.0 mM benzamidine, and 0.1% (w/w) 2-mercaptoethanol), and cells were lysed using a French press (1000–1500 psi). The lysate was heated in boiled water for 10 min and was then subjected to centrifugation at 20,000 × g to remove insoluble material. The supernatant was loaded onto a DEAE-cellulose column (100 ml); the column was washed, and bound proteins were eluted using a linear gradient from 0.00 to 0.6 M NaCl (in buffer B, 1000 ml total volume). Fractions containing inhibitor-2 were pooled and concentrated to a volume of 5 ml by ultrafiltration using a YM-10 membrane. The concentrated sample was diluted with 10 ml of buffer B (20 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 1.0 mM dithiothreitol) and loaded onto a Blue-Sepharose column (40 ml). The column was washed, and bound protein was eluted with a linear gradient from 0.00 to 1.0 M NaCl and was then eluted with buffer B (500 ml total volume). Fractions containing inhibitor-2 were pooled and diluted by addition of 1 volume of buffer B. The sample was loaded onto a Mono-Q column (Amersham Pharmacia Biotech 10/10); the column was washed, and proteins were eluted with a linear gradient from 0.18 to 0.6 M NaCl (in buffer A). Fractions containing purified inhibitor-2 were pooled, dialyzed against water, and lyophilized. Wild-type inhibitor-2 and all mutants were purified to greater than 95% homogeneity as judged by SDS-PAGE (see Fig. 3). The yield of each mutant was similar to that of the wild-type protein (2–5 mg/liter). In general the binding affinity of inhibitor-2 mutants to Blue-Sepharose was proportional to their inhibition potencies.

Preparation of I2-(t1–84) and I2-(85–204)—TT-tagged inhibitor-2 was cleaved with 2-nitro-5-thiocyano benzoic acid using a modification of the method described by Girault et al. (22). I2-(11–84) eluted at ~0.2 M NaCl and was separated from I2-(85–204) and intact T7-tagged inhibitor-2 (eluted at ~0.3 M NaCl). Fractions containing I2-(85–204) and the intact protein were pooled, dialyzed against water, and lyophilized. The protein was dissolved in 1 ml of buffer B and loaded onto the Blue-Sepharose column. Intact inhibitor-2 bound to the column, whereas I2-(85–204) eluted in the flow-through. Fractions containing purified proteins were pooled, dialyzed against water, and lyophilized.

Preparation of Wild-type and Mutant DARPP-32—Vector construction, plasmid transformation, and protein expression were performed essentially as described (23). The rat cDNA in pET3a was used as a template for site-directed mutagenesis using polymerase chain reaction. Deletion, insertion, and amino acid substitution mutants were amplified using appropriate primers from 100 ng of DARPP-32 cDNA (all oligonucleotides were synthesized by Operon). The amplified DNA was gel purified, digested with NdeI and BamHI, and subcloned for the appropriate part of the DARPP-32 cDNA in the pET3a vector. All mutations were confirmed by DNA sequencing.

DARPP-32 mutants were purified by heat treatment, anion-exchange chromatography using DEAE-cellulose, gel filtration using Sephacryl S-200, and fast protein liquid chromatography using a Mono-Q column. Wild-type DARPP-32 and all mutant proteins were purified to greater than 95% homogeneity as judged by SDS-PAGE (data not shown). Wild-type and mutant DARPP-32 was phosphorylated stoichiometrically by cAMP-dependent protein kinase, and phospho-DARPP-32 was purified using a Mono-Q column as described (17).

PP1 Assays—PP1 was assayed using [γ-32P]phosphorylase a as a substrate essentially as described (28). Assay mixtures (final volume 30 μl) contained 50 mM Tris-HCl buffer (pH 7.4), 15 mM MgCl2, 5 μM ATP, 0.01% (w/w) Brij 35, 0.3 mg/ml bovine serum albumin, 5 mM caffeine, 10 μM [γ-32P]phosphorylase a, various protein inhibitors, and PP1. For inhibitor-2 assays, PP1 and inhibitor-2 were preincubated at 30 °C for 10 min. Dephosphorylation reactions were initiated by the addition of substrate, and reactions were carried out for 10 min at 30 °C. Antagonist peptides and inhibitor proteins were premixed before addition of PP1. All reactions were performed in duplicate. All experiments were performed at least two times, with typical errors being less than 20% of the mean. PP1 Overlay Assay—Binding of PP1 to wild-type and mutant inhibitor-2 was analyzed using a PP1 overlay technique essentially as described (24). Briefly, proteins were separated by SDS-PAGE (using the method of Laemmli, 12.5% acrylamide) and transferred to nitrocellulose filters. The nitrocellulose filters were incubated with a buffer containing 10 mM Tris-HCl (pH 7.4), 2% (w/v) dried milk, 0.1% Tween 20. Filters were washed with phosphate-buffered saline containing 0.2% Nonidet P-40 and then incubated with phosphate-buffered saline/Nonidet P-40 containing 0.5 μg/ml recombinant PP1 for 2 h at 4 °C. Filters were washed with phosphate-buffered saline/Nonidet P-40, and bound PP1 was detected using antibody to PP1 as described (25).

RESULTS

Characterization of Inhibition of PP1 by Site-directed Mutants of DARPP-32—Our previous studies have provided support for a model in which two distinct subdomains in DARPP-32 interact with PP1. Subdomain 1 contains the phospho-Thr34 region (FRRRTPLMF; residues 29–38), and subdomain 2 includes a short stretch of residues at the NH2 terminus of the protein (RKKIQF; residues 6–11). Site-directed mutagenesis of full-length DARPP-32 was therefore used to assess the role of individual amino acids in subdomains 1 and 2 and also the relationship between the two subdomains. Each mutant was phosphorylated stoichiometrically by cAMP-dependent protein kinase, and the inhibitory potencies of the various phosphoproteins was measured.
were omitted for clarity (see Table I). Closely agreeing duplicate measurements. Results for some mutants DARPP-32. PP1 was assayed using 10 μM [γ-32P]phosphorylase a as substrate. Phosphatase activity is expressed as percent of activity measured in the absence of addition of phospho-DARPP-32. A, inhibition of PP1 by selected subdomain 2 mutants. B, inhibition of PP1 by selected subdomain 1 mutants. The points shown are the average of closely agreeing duplicate measurements. Results for some mutants were omitted for clarity (see Table I).

Within subdomain 2, deletion of amino acids 6–9 (D32-(Δ6–9)) or replacement of these residues (D32-(R6S,K7Q,R8Q,19S)) resulted in a large increase in the IC₅₀ for inhibition of PP1 (Fig. 1A and Table I). Further deletion of residues 10 and 11 (D32-(Δ6–11)) resulted in an additional increase in the IC₅₀. Within this region, mutation of Lys-7, but not Lys-8, resulted in a small but significant increase in the IC₅₀. Mutation of Ile-9 to glycine resulted in a large increase in the IC₅₀; however, mutation of this residue to alanine had little effect. Mutation of Phe-11 to alanine resulted in a large increase in IC₅₀; however, further deletion of residues 10–11 caused a large increase in IC₅₀. Further removal of NH₂-terminal residues had little additional effect.

An overlay technique was used to assess the direct interaction of the inhibitor-2 mutants with PP1. Binding to PP1 was determined from results shown in Fig. 1 and data not shown. The effect of selected mutants was also analyzed using dephospho-proteins. The inhibition of each phospho-mutant was measured relative to wild-type phospho-DARPP-32, and the inhibition of each dephospho-mutant was measured relative to wild-type dephospho-DARPP-32. ND, not determined.

| Inhibitor | Phospho-DARPP-32 | Dephospho-DARPP-32 |
|-----------|------------------|--------------------|
|           | IC₅₀ | Relative inhibition | IC₅₀ | Relative inhibition |
| D32-(1–202)-wt | 1.4  | 100 % | 1600 | 100 % |
| D32-(Δ6–9) | 45 | 3.1 | 12,500 | 13 |
| D32-(R6S,K7Q,R8Q,19S) | 50 | 2.8 | 10,000 | 16 |
| D32-(Δ6–11) | 110 | 1.3 | ND | ND |
| D32-(K7E) | 5.0  | 28 | ND | ND |
| D32-(K8E) | 0.9 | 155 | ND | ND |
| D32-(I9G) | 41 | 3.4 | ND | ND |
| D32-(H9A) | 2.0 | 70 | ND | ND |
| D32-(F11A) | 48 | 2.9 | ND | ND |
| D32-(F11W) | 0.9 | 155 | ND | ND |
| D32-(Δ15–18) | 12 | 12 | 4200 | 38 |
| D32-(ins15/AAA/16) | 1.7 | 82 | ND | ND |
| D32-(L20A,V25A,I28A) | 19 | 7.4 | ND | ND |
| D32-(P33G,P35G) | 37 | 3.8 | 2200 | 73 |
| D32-(R30A) | 1.8 | 78 | 2600 | 62 |
| D32-(R29A) | 2.9 | 48 | 3300 | 49 |
| D32-(R29A,R30A) | 9.4 | 15 | 2300 | 70 |

The role of certain of the amino acids that link subdomains 1 and 2 was also investigated. Combined mutation of several hydrophobic amino acids that might have formed an amphipathic α-helix (Leu-20, Val-25, and Ile-28; D32-(L20A,V25A,I28A)) resulted in an increase in IC₅₀. Deletion of residues 15–18 (D32-(Δ15–18)) also resulted in an increase in IC₅₀. In contrast, insertion of three alanine residues between residues 15 and 16 (D32-(ins15/AAA/16)) had no effect. The dephospho-form of DARPP-32 binds to PP1 and inhibits enzyme activity, although with an IC₅₀ in the micromolar range. The ability of dephosphoforms of some of the mutants to inhibit PP1 was therefore also analyzed (Table I). Deletion or substitution of residues 6–9 resulted in significant increases in IC₅₀. Deletion of residues 15–18 also resulted in a small increase in IC₅₀. In contrast, dephospho-D32-(ins15/AAA/16) exhibited a slight decrease in IC₅₀. Mutation of amino acids within subdomain 1 resulted in only small increases in IC₅₀.

Characterization of Inhibition of PP1 by Truncation Mutants of Inhibitor-2—In an attempt to identify regions of inhibitor-2 that are involved in binding to PP1 and inhibition of enzyme activity, a series of truncation mutants were expressed as recombinant proteins. In addition, wild-type inhibitor-2 was chemically cleaved with 2-nitro-5-thiocyanobenzoic acid to produce two large fragments, I2-(t1–84) and I2-(85–205), that were also analyzed. Dose-response curves for the inhibition of PP1 by the various proteins were obtained (Fig. 2A), and the IC₅₀ values were calculated (Table II). Under the assay conditions used, the IC₅₀ for inhibition of PP1 by wild-type inhibitor-2 was ~1 nM, a result very similar to that obtained in previous studies (5). Deletion of up to 105 amino acids at the COOH terminus (I2-(1–89)) had little effect on the IC₅₀, although further deletion of 15 residues (I2-(1–84)) resulted in a small but significant increase in IC₅₀. Deletion of the first 8 amino acids at the NH₂ terminus had no effect on the IC₅₀, however, deletion of residues 9–13 caused a large increase in IC₅₀. Further removal of NH₂-terminal residues had little additional effect.

An overlay technique was used to assess the direct interaction of the inhibitor-2 mutants with PP1. Binding to PP1 was observed for full-length inhibitor-2 and for I2-(1–164), I2-(1–172), I2-(1–180), I2-(9–204), I2-(9–164) (Fig. 3), and I2-(1–159) (data not shown). However, further deletion of residues 9–13 (I2-(14–204)) or more amino acids from the NH₂ terminus (I2-(19–204), I2-(39–204), I2-(58–204), I2-(85–204)), or further deletion of residues 141–159 (I2-(1–140)) or more amino acids from the COOH terminus (I2-(1–120), I2-(1–99)) resulted in a
loss of PP1 binding as measured by this assay. In preliminary studies the ability of the various inhibitor-2 truncation mutants to form a stable complex with PP1 (cf. Ref. 5) was also investigated (data not shown). PP1 formed a complex with I2-(1–180), I2-(1–172), I2-(1–164), I2-(1–158), I2-(1–140), I2-(1–109), I2-(9–204), I2-(14–204), and I2-(19–204) but not with I2-(39–204) or I2-(58–204). Additional studies will be necessary to measure the relative affinities of these interactions and to measure the ability of the complexes to be reactivated by phosphorylation by GSK-3.

Characterization of Inhibition of PP1 by Site-directed Mutants of Inhibitor-2—The results obtained using truncated fragments of inhibitor-2 suggested that amino acids at both the NH2- and COOH termini of the protein were important for binding to PP1 and inhibition of enzyme activity. Amino acids 9–13 (PIKGI) appeared to be the most important for inhibition, and the contribution of individual amino acids in this region was further assessed by site-directed mutagenesis (Fig. 2B and Table II). Mutation of Ile-10 (I2-(I10G)) had a small but significant effect on inhibition of PP1. Mutation of either Lys-11 (I2-(K11E)), or Ile-13 (I2-(I13G), I2-(I13A)) resulted in large increases in the IC50. Notably, substitution of bulky hydrophobic amino acids for Ile-13 (I13F) and I2-(I13W) caused a larger increase in IC50 than that of glycine or alanine. Combined mutation of Ile-10 with Lys-11 or Ile-10 with Ile-13 resulted in little additional loss of inhibitory potency relative, respectively, to that of mutation of Lys-11 or Ile-13 alone. However, combined mutation of Lys-11 with Ile-13 resulted in a more than additive loss of inhibitory potency relative to that of mutation of Lys-11 or Ile-13 alone.

The potential contribution of several other residues in inhibitor-2 was also assessed. Mutation of Thr-72 (I2-(T72A)), the residue phosphorylated by GSK-3, had no effect on inhibitory potency. This is the same result as described in a previous study (5). The results shown in Fig. 3 and data not shown) indicated that residues 141–159 appeared to be important for inhibition. Comparison of the Inhibition of PP1 by Site-directed Mutants of Inhibitor-2 and DARPP-32—The results described above indicated that basic and hydrophobic amino acids in the NH2-terminal domains of both inhibitor-2 (residues 11–13) and DARPP-32 (residues 7–11) were critical for inhibition of PP1. However, comparison of the results from site-directed mutagenesis suggested that there may be differences between the binding motifs of the two proteins. Therefore, we compared the ability of synthetic peptides, based on amino acid sequences containing the two bind-
DARPP-32 and Inhibitor-2

**DISCUSSION**

The results from the present study suggest that phospho-DARPP-32 and inhibitor-2, two heat-stable inhibitors of PP1, interact with the catalytic subunit via different mechanisms that involve the interaction of common and distinct subdomains within the two inhibitors. Interaction of PP1 with either inhibitor requires at least two subdomains within each protein binding motifs, to antagonize the interactions of the proteins with PP1.

I2-(6–20), added at a fixed concentration (25 μM), increased the IC₅₀ for inhibitor-2 by more than 20-fold (Fig. 4A) but had no significant effect at concentrations as high as 100 μM on the ability of phospho-DARPP-32 to inhibit PP1 (Fig. 4B). I2-(6–20) had no significant effect on PP1 activity at 25 μM and a small inhibitory effect at 100 μM (data not shown). However, dephospho-D32-(6–38c), which increased the IC₅₀ for phospho-DARPP-32 by greater than 25-fold (Fig. 5B), also increased the IC₅₀ for inhibitor-2 by more than 5-fold (Fig. 5A). A similar result was obtained using a peptide derived from the PP1 binding domain of M110, the myosin-binding subunit of PP1, that contains a binding motif related to that found in subdomain 2 of DARPP-32 (8, 12, 13) (Fig. 6, A and B), and a smaller peptide derived from DARPP-32 (D32-(4–14)) (data not shown). Dephospho-D32-(6–38c) increased the IC₅₀ for I2-(1–120) by greater than 100-fold (Fig. 7A) but had no significant effect on the IC₅₀ for I2-(85–204) (Fig. 7B). Thus inhibitor-2 appears to also contain a second region that binds or overlaps the part of PP1 that interacts with DARPP-32 and other PP1-binding proteins. The potential contribution of residues 131–155 of inhibitor-2 was also assessed by peptide competition studies (data not shown). I2-(131–155) (10 or 100 μM) had no significant effect on the ability of phospho-DARPP-32 to inhibit PP1, although this peptide increased the IC₅₀ for inhibitor-2 by ~3-fold.

**FIG. 3. Binding of inhibitor-2 mutants to PP1.** A series of inhibitor-2 mutants were expressed in E. coli and purified to homogeneity. Approximately equal amounts of each mutant (~2 μg) were separated by SDS-PAGE (12% acrylamide), and the gel was stained with Coomasie Brilliant Blue (lower panel). In a separate analysis, approximately equal amounts of each protein (~1 μg) were separated by SDS-PAGE, and the proteins were transferred to nitrocellulose. The nitrocellulose membrane was incubated with purified recombinant PP1, and bound PP1 was detected using a PP1 antibody and autoradiography as described under “Experimental Procedures” (upper panel).

**FIG. 4. Effect of I2-(6–20) on inhibition of PP1 by inhibitor-2 and phospho-DARPP-32.** A, PP1 activity was measured in the absence (○) or presence (●) of 25 μM I2-(6–20) and the indicated concentrations of inhibitor-2. B, PP1 activity was measured in the absence (○) or presence (●, 25 μM; □, 100 μM) of I2-(6–20) and the indicated concentrations of phospho-DARPP-32. 25 μM I2-(6–20) inhibited PP1 activity by less than 10%; 100 μM I2-(6–20) inhibited PP1 activity by 20%. In this and Figs. 5–7, PP1 activity is expressed as percent of the activity measured in the absence of inhibitor peptide. In each case, the points shown are the average of closely agreeing duplicate measurements, and experiments were performed at least twice with similar results being obtained.

(Fig. 8A). In the case of phospho-DARPP-32, these two subdomains are included between residues 6 and 38. Residues close to, and including the phosphorylated form of Thr-34, define subdomain 1, and a short motif between residues 7 and 11 (KKI(F) defines subdomain 2. In the case of inhibitor-2, a larger part of the molecule (residue 9 to approximately residue 99) is required for inhibition. A short NH₂-terminal amino acid motif (IKGI, subdomain 3) is located between residues 10 and 13 of inhibitor-2. Subdomain 2 in DARPP-32 and subdomain 3 in inhibitor-2 both include basic and hydrophobic amino acids that are important for inhibition of PP1. However, these motifs are unrelated to each other and bind to different regions of the catalytic subunit that are both removed from the active site of the enzyme (Fig. 8B). Inhibitor-2 also contains a region between residues 15 and 54 that binds or overlaps the part of PP1 that interacts with subdomain 2 of DARPP-32. For phospho-DARPP-32, inhibition of PP1 requires the interaction of phospho-Thr-34 with the active site of the enzyme or with residues very close to the active site. In the case of inhibitor-2, inhibition of PP1 presumably requires the interaction of residues between 15 and 84 with the active site of the enzyme or with residues close to the active site.

This study extends our investigations of the role of subdomains 1 and 2 of phospho-DARPP-32 in the interaction with
Our previous studies had indicated that phospho-Thr-34 is essential for potent inhibition of PP1; notably, a peptide containing phosphoserine in place of phosphothreonine was ineffective as an inhibitor (16). Mutation of residues in the active site of PP1 had a parallel effect on the inhibitory potency of phospho-DARPP-32 and a variety of toxins that are known to interact with the active site (17). In addition, mutation of residues away from the active site of PP1 was able to influence the ability of the enzymes to either be inhibited by phospho-DARPP-32 or alternatively to dephosphorylate phospho-Thr-34 (17). Whereas the underlying structural basis for the latter result is not known, these results suggest in native PP1 that phospho-Thr-34 interacts with residues close to or in the active site in a manner in which it cannot be dephosphorylated. The fact that mutation of Pro-33 and Pro-35 reduces the inhibitory potency of phospho-DARPP-32 suggests that these two residues that flank phospho-Thr-34 may be involved in this interaction.

The results also extend our knowledge of the precise role played by specific amino acids within the basic/hydrophobic motif that is found in subdomain 2 of DARPP-32 and within subdomains of a growing number of PP1-binding proteins (4, 8, 9, 12, 27–32). The exact sequence of the motif is not identical, but one or more basic amino acids is followed by two hydrophobic residues separated by a variable amino acid. The first of the two hydrophobic amino acids is either valine or isoleucine, and the second hydrophobic residue is phenylalanine (11–15). The molecular basis for the interaction of this BB(V/I)XF motif with PP1 has recently been determined using x-ray crystallography (12). Six residues (RRVSFA) of a 13-residue peptide containing the PP1-binding domain of GM are ordered in the crystal structure and interact in an extended manner with a hydrophobic channel situated on the side opposite from that of the active site of PP1. Interactions are found between the side chains of the valine and phenylalanine in the peptide and solvent-exposed hydrophobic side chains in PP1. Electrostatic interactions are also found between the two arginine residues in the peptide and acidic residues in PP1.

In the present study, mutation of Ile-9 to glycine reduced the inhibitory potency of phospho-DARPP-32. Mutation to alanine was largely accommodated, suggesting that there is some flexibility in the identity of the hydrophobic amino acid at this position. Mutation of Phe-11 to alanine reduces the inhibitory potency of phospho-DARPP-32; however, phospho-DARPP-32 in which Phe-11 was mutated to tryptophan retained full inhibitory potency. A random peptide library analysis has indicated that tryptophan may substitute for phenylalanine (15). In addition, our recent studies have indicated that tryptophan is the residue found in this position in the PP1 binding motif of the nuclear targeting, PP1-binding subunit, PNUTS (9).2 Mutation of Lys-7 (to glutamate) had a relatively small effect on inhibitory potency. Unexpectedly, mutation of Lys-8 (to glutamate) had no effect.

2 Y.-S. Kwon, P. Allen, M. Konarsha, P. Greengard, and A. C. Nairn, unpublished results.
the two proteins. Circular dichroism studies of DARPP-32\(^3\) and inhibitor-1 (33) have indicated that both proteins are largely disordered in structure. These hydrophobic residues could therefore interact in an extended conformation with surface-exposed hydrophobic amino acids in PP1. Alternatively, the region between the subdomains of DARPP-32 may have the ability to form an amphipathic \(\alpha\)-helix upon binding to PP1.

Assuming that Phe-11 of DARPP-32 interacts with PP1 in the same way as Phe-68 of the G\(_M\) peptide (12), and phospho-Thr-34 interacts with the active site of the enzyme, there are several alternative ways in which intervening residues could interact with PP1. We have previously suggested, based on initial modeling studies, that the basic amino acid side chains in residues 29–32 might interact with several acidic amino acids present in a groove close to the active site of PP1 (34). However, this model is not supported by the present results that indicated that Arg-29 and Arg-30 did not make a major contribution to the interaction of DARPP-32 with PP1. In addition, mutation of several of the acidic amino acids in PP1, either singly or in combination, did not reduce the inhibitory potency of phospho-DARPP-32 (17). Given that many of these acidic residues are found only in the catalytic subunit of PP1, but not PP2A and PP2B, it remains an attractive possibility that they play a role in binding other PP1-binding proteins. Alternative modes of binding for phospho-DARPP-32 could include the following: interaction 1) with residues in the COOH-terminal groove that also emanates from the active site of the enzyme, or 2) with the \(\beta_1\)-\(\beta_3\) loop situated above the active site in a manner similar to that of the autoinhibitory segment of PP2B (35).

The results obtained from the present studies indicate that the first half of inhibitor-2 (approximately residues 9–99) retains the same inhibitory qualities as the wild-type protein. A subdomain including residues 10–13 defines a novel PP1 binding motif (IKG) that is distinct from that identified in subdomain 2 of DARPP-32 (KKIQF). These results extend previous studies that had indicated an important role for the NH\(_2\)-terminal 35 residues of the protein in inhibition of PP1 (5). In addition to the novel PP1 binding region, residues 15–84 of inhibitor-2 also contain a second region that binds or overlaps the part of PP1 that interacts with DARPP-32, and these distinct but overlapping modes of binding of DARPP-32 and inhibitor-2 to PP1 are consistent with previous kinetic studies (36). Analysis of the primary structure of inhibitor-2 had failed to identify any obvious similarity to DARPP-32, inhibitor-1, or other PP1-binding proteins (11, 18, 37). Inhibitor-2 is, however, similar in sequence to the Glc8 protein, an apparent functional homolog identified in Saccharomyces cerevisiae (38). The level of amino acid identity in inhibitor-2 and Glc8 is low (28% over 190 residues), but there are several short stretches of the two proteins that are highly conserved, including the region around Thr-72 (of inhibitor-2). Based on a previous alignment of the amino acid sequences of inhibitor-2 and Glc8 (38), residues 10–14 of inhibitor-2 (IKG) are IPGL in Glc8 (residues 48–51). Interestingly, in inhibitor-2 the sequence KKSQKW (residues 41–46) is conserved as EERVQW in Glc8 (residues 88–93), the latter sequence in Glc8 containing a sequence equivalent to the PP1 binding motif found in subdomain 2 of DARPP-32. Little is known about the biochemical properties of Glc8, but it is possible that these regions of inhibitor-2 and Glc8 represent functionally conserved PP1 binding regions that have diverged through evolution.

An important question raised by these and previous studies relates to whether part of inhibitor-2 interacts with the active

\(^3\) H. C. Hemmings, unpublished observations.
site of PP1. Kinetic studies indicate that inhibitor-2 is a competitive inhibitor when phosphorylase a is used as a substrate (36). Inhibitor-2 also competes with binding of okadaic acid and microcystin to PP1 (39, 40), and both of these toxins have been found to bind to the active site of PP1 (17, 34). Moreover, mutation of amino acids between residues 76 and 85 of inhibitor-2 resulted in PP1-inhibitor-2 complexes that showed high constitutive activity, in contrast to the normally inactive complex obtained with wild-type proteins (5). These various studies all point to the conclusion that part of inhibitor-2, including residues 76–85, either interacts directly with the active site of PP1 or with residues close to the active site in a manner that blocks binding of peptide substrate (see Fig. 8F).

The overlay analysis suggested that residues 9–14 contribute to a high affinity interaction between inhibitor-2 and PP1. Whereas our studies focused on the mechanism of inhibition and not of complex formation or inactivation of PP1, preliminary results indicated that residues 1–99 of inhibitor-2 were able to form a stable complex with PP1. Potentially, binding of residues 9–14 to PP1 might represent a first step in the interaction of inhibitor-2 with PP1 that ultimately results in complex formation and inactivation following the binding of other parts of the two proteins. The overlay analysis also suggested that residues 141–159 of inhibitor-2 are involved in binding to PP1. However, deletion and site-directed mutagenesis and peptide competition studies all indicate that residues 141–159 are not involved in inhibition of PP1. Notably, this region is well conserved in Glc8, so it is likely to play some important functional role. Previous studies have indicated that residues 146–204 are important for reactivation of PP1 (5), and it therefore seems likely that the high affinity interaction between residues 141 and 159 and PP1 is involved in complex formation and/or reactivation of the enzyme. Recent studies have indicated that inhibitor-2 is present in both the cytosol and nucleus and that the expression of the protein is regulated in a cell cycle-dependent manner (41, 42). It is not known if all of the cellular inhibitor-2 is complexed with PP1; however, the localization of the protein appears to be controlled by both nuclear localization and nucleus export signals that are located between residues 140 and 160 (42). The interaction of inhibitor-2 with PP1 via this region may therefore modulate, or be modulated, by the trafficking of inhibitor-2 into and out of the nucleus.

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Protein Phosphatase-1, DARPP-32, and Inhibitor-2

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