A Novel Phosphoprotein Inhibitor of Protein Type-1 Phosphatase Holoenzymes†

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ABSTRACT: Control of protein phosphatases is now understood to depend on binding to a variety of regulatory or targeting subunits to form holoenzymes with restricted localization and substrate specificity. In addition, the catalytic subunits of both type-1 and type-2 phosphatases bind specific inhibitor proteins. Here, we report discovery of a new inhibitor protein called PHI-1 that is specific for type-1 protein phosphatase (PP1). Recombinant tagged PHI-1 was phosphorylated by protein kinase C at two sites, one a Ser and one a Thr; phosphorylation enhanced inhibitory potency 50-fold. Mutation of Thr57 to Ala gave a protein phosphorylated only on Ser, without change in inhibitory activity, indicating that phosphorylation of Thr57 was required for full activity. Immunoblotting showed that PHI-1 was expressed in most animal tissues and several cell lines, and a second larger protein called PHI-2 was present in different muscles, especially cardiac muscle. Unlike any other known inhibitor, PHI-1 inhibited the myosin- and glycogen-associated holoenzyme versions of PP1 as well as the monomeric catalytic subunit of PP1. Discovery of PHI-1 and PHI-2 opens new possibilities for regulation of PP1 via phosphorylation-dependent signaling pathways.

Inhibitor proteins for protein phosphatases have been known for almost 25 years (*1*) and have proved useful as a means of distinguishing type-1 from type-2 Ser/Thr phosphatases (*2*). Until recently, three heat-stable proteins specific for protein type-1 phosphatase (PP1)[1] were known: inhibitor-1 (I-1), dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32), and inhibitor-2 (Inh2). I-1 and DARPP-32 are present in various animal tissues and enriched in specific regions of the brain (*3*). Phosphorylation of a Thr residue by cAMP-dependent protein kinase increases the inhibitory potency of these proteins by 1000-fold, making them effective at sub-nanomolar concentrations (*4, 5*). Inhibitor-2 binds PP1 catalytic subunit (PP1C) with nanomolar affinity, and inhibits, then slowly converts the phosphatase to an inactive form (*6*). The Inh2 undergoes multisite phosphorylation, in particular at a Thr residue that triggers reversible conversion of the PP1C to an active form. Expression and localization of Inh2 fluctuates during the cell growth cycle, peaking at the S phase and mitosis (*7*) and going from cytoplasmic during G1 phase to nuclear during S phase (*8*). However, none of these inhibitors are effective against PP1 bound to regulatory/targeting subunits, though extended preincubation of the phosphatase under certain conditions can render it sensitive to the inhibitors. There is an abundance of regulatory subunits, and it is now thought that essentially all the PP1 in cells is present in holoenzymes. This would limit the physiological action of the inhibitor proteins to conditions when PP1C dissociates from the holoenzymes.

More recently, three additional PP1 inhibitor proteins have been discovered and characterized. There is one report of a protein called inhibitor-3 (Inh-3) (*9*). Inh-3 is in mammals, with proteins of related sequences in *Saccharomyces cerevisiae*, and *Caenorhabditis elegans*. This potent inhibitor does not require phosphorylation, and is specific for PP1 compared to PP2A, but was not tested against holoenzymes of PP1. Another protein, exclusively expressed in brain, is phosphorylated by cGMP-dependent protein kinase, and the phosphorylated protein functions as an inhibitor of PP1. This phosphorylation-dependent inhibitor has been called G-substrate (*10*). Another new PP1 inhibitor is called CPI-17, for PKC-dependent phosphatase inhibitor of 17 kDa. CPI-17 was purified from and is only expressed in smooth muscle (*11, 12*). It inhibits PP1C with sub-nanomolar potency, but only after phosphorylation of a single Thr residue by PKC. Most striking, CPI-17 inhibits myosin-associated PP1, which is bound to a regulatory M subunit, a property heretofor not reported for any PP1 inhibitor protein (*13*). Thiophosphorylated CPI-17 causes contraction of skinned smooth muscle (*14, 15*), suggesting that it might have an important role in Ca^{2+}-sensitization of smooth muscle.

Because CPI-17 could inhibit a holoenzyme form of PP1, we reasoned that inhibition of PP1 holoenzymes occurred in cells as a fundamental facet of signaling processes. If so, then a protein resembling CPI-17 must be present in tissues other than smooth muscle. Here, we report finding a DNA sequence that encodes an open reading frame for a protein resembling CPI-17, called PHI. PHI requires phosphorylation on a Thr residue for full potency, and it inhibits forms of PP1 that are holoenzmes composed of members of the M subunit and G subunit families. Thus, PHI is the first protein

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[1] Abbreviations used: PKC, protein kinase C; CPI-17, the PKC-dependent phosphatase-1 inhibitor of 17 kDa; H6[44]PHI-1, a fusion protein of mouse PHI-1 with a 44 residue N-terminal extension including a His_6 tag; PNG, phospholipase Cβ neighboring gene; PHI-1, phosphatase holoenzyme inhibitor starting with the second Met encoded by PNG; PHI-2, phosphatase holoenzyme inhibitor starting with the first Met encoded by PNG; ORF, open reading frame; PP1, protein type-1 phosphatase; PP1C, catalytic subunit of PP1.

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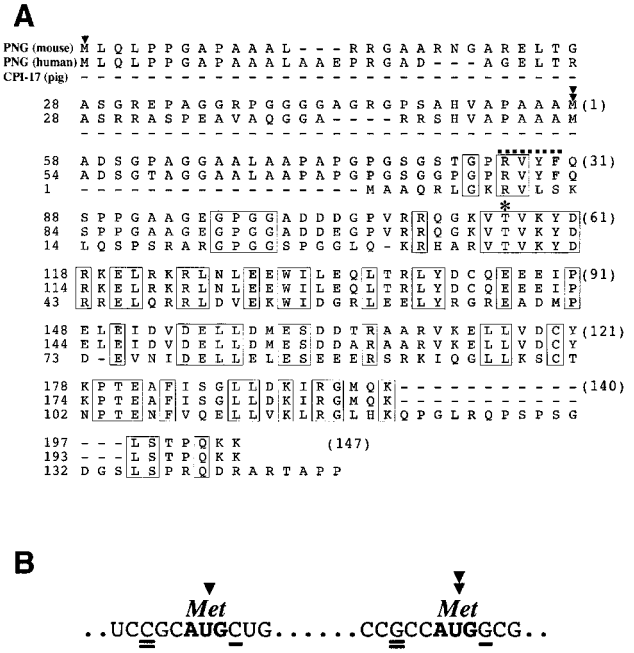

FIGURE 1: (A) Amino acid sequence alignment of phospholipase-C neighboring gene (PNG) product with CPI-17. Deduced amino acid sequence of PNG from mouse (*17*) and human (*25*) were aligned with that of porcine CPI-17 (*12*). Residues conserved among three sequences are enclosed in boxes. Hyphens indicate insertions to give maximum matching. An asterisk is put over Thr-38 position of CPI-17 that is phosphorylated to increase potency of inhibition. A broken line indicates the RVxF motif common to PP1C binding proteins. Single and double arrowheads indicate probable initiation Met for translation of PHI-2 and PHI-1, and residue numbers from both Met are indicated to the left and right sides, respectively. (B) mRNA sequence around potential initiation codon of PNG. Single and double arrowheads indicate initiation Met of PHI-2 and PHI-1 in panel A, respectively. The bases at positions −3 and +4 (double and single underlines) determine preference for initiatation of translation.

that inhibits multiple holoenzyme forms of PP1, and its phosphorylation may link various signaling pathways to regulation of PP1.

EXPERIMENTAL PROCEDURES

Recombinant PHI-1 Proteins. A PNG cDNA clone from mouse embryo EST library was purchased from American Type Culture Collection (GenBank Accession #W33783). The cDNA sequence was verified by dideoxy sequencing *(16)*, showing this was identical to mouse PNG cDNA within the PHI-1 region (Figure 1) (*17*). PHI-1 segment was excised by digestion with *Nco*I and *Bam*HI and was ligated at corresponding sites of pET30b (Novagen) vector. The vector introduces 44-residues N-terminal tag, including a 6x His segment (H6[44]). H6[44]-PHI-1 was prepared as described previously, with minor modifications (*12*). Briefly, H6[44]-PHI-1 was induced by addition of 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) in 500 mL of *Escherichia coli* BL21(DE3) pLysS culture that was transformed with pET30b-PHI-1. Bacterial extract was obtained by a freeze-and-thaw method. The extract was incubated for 10 min in boiling water and centrifuged to remove coagulated protein. H6-[44]-PHI-1 in the supernatant of the boiled extract was bound to Ni^{2+}-NTA resin. The eluate from Ni^{2+}-NTA resin was concentrated to about 1 mL by an ultrafilter membrane unit (Centricon 10), prior to loading onto a 1.4 × 42 cm column of Sephadex G-75 Superfine (Pharmacia). Gel filtration was carried out at flow rate of 5 mL/h with 50 mM ammonium bicarbonate including 0.1% 2-mercaptoethanol, and 1 mL fractions were collected. Protein elution was monitored by absorbance at 280 nm. Typical yield was about 3 mg of H6-[44]-PHI-1 from a 500 mL culture. A point mutation at Thr57 was carried out using QuickChange Site-Directed Murtagenesis Kit (Stratagene), with wild-type pET30b-PHI-1 as a template. A pair of primers including mismatch bases (in italics), GTCGTACTTGACGG*C*GAGCTTCCCTTG and CAAGGGAAGGTC*G*CCGTCAAGTACGAC were used. T57A-H6[44]-PHI-1 was purified as described above. The H6[44] tag was removed from 5 μg of purified H6[44]-PHI-1 by digestion with 0.02 U of recombinant enterokinase (Novagene) for 16 h at 30 °C. Enterokinase cleaves 2 residues N-terminal to initial Met of PHI-1.

Preparation of Phospho-H6[44]-PHI-1. Phosphorylation was carried out for 120 min under condition of 0.2 mg/mL H6[44]-PHI-1, 2 μg/mL PKC delta, 0.1 mM ATP, 10 mM magnesium acetate, 1 μM microcystin LR, 1 mM benzamidine, 1 mM dithiothreitol, and 25 mM MOPS-NaOH pH 7.0, at 30 °C. The reaction was terminated by incubation for 5 min at 100 °C, prior to loading onto Sephadex G-50 column (1 mL) to exchange the protein into 10 mM MOPS-NaOH pH 7.0. Stoichiometric phosphorylation was confirmed by urea-PAGE.

Other Proteins. Myosin-bound phosphatase was prepared from pig aorta as described previously (*11*). The glycogen-bound phosphatase, PP1C and phosphorylase b all were prepared from rabbit skeletal muscle (*18*−*20*). PP2A and PP2B was prepared from human red blood cells and pig brain, respectively (*12*, *21*). Phosphorylated smooth muscle myosin LC20 and phosphorylase a were prepared using [γ-^{32}P]ATP (*13*). A truncated form of protein kinase C delta (PKC), which loses phorbol ester dependency, was isolated from pig aorta (Eto, M. unpublished).[2]

Immunoblotting. Total proteins of rat tissues were extracted with 5 vol of 20 mM Tris-HCl pH 7.4, including 2% SDS, 10% 2-mercaptoethanol, 5% glycerol, 0.2 mM Pefabloc (Boerhinger-Mannheim), and 1 mM benzamidine. Cultured cells in a 100 mm dish were lysed with 0.5 mL of 50 mM Tris-HCl, pH 8.0, including 1% IGEPAL CA-630, 0.1 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM Pefabloc, 5 μg/mL leupeptin, and 0.1% 2-mercaptoethanol, and then 10 μg of proteins in the supernatant were resolved by SDS-PAGE, carried out according to Porzio and Pearson (*22*). Proteins in the gel were transferred onto a nitrocellulose filter (0.2 μm BioRad), and nonspecific sites were blocked with 5% nonfat milk. Anti-PHI-1 antibody was produced in rabbits with H6[44]-PHI-1 fusion protein as an antigen and was purified using affinity resin conjugated with PHI-1 purified after enterokinase cleavage. Anti-CPI-17 antibody (*13*) was further purified with resin conjugated with CPI-17 recovered after cleavage by enterokinase. Specific proteins were visualized by use of chemiluminescence reagent (NEN). Both PHI-1 and PHI-2 staining was completely blocked by preincubation of anti PHI-1 antibody with 0.2 μM H6[44]-PHI-1 (data not shown).

Other Methods. Phosphatase assay was performed as described previously (*12*, *13*). Conditions used were 25 mM

[2] Eto, M., unpublished results.

MOPS-NaOH pH 7.0 including 5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.4 mM Pefabloc, for 10 min at 30 °C. Okadaic acid (1 nM) was added to PP1C and PP2B assay mixtures. PP2B assay was carried out in the presence of 0.2 mM $CaCl_2$, 20 μg/mL calmodulin and 1 mM $MnCl_2$. Reaction was initiated by addition of the phosphatase preparation. Urea-PAGE was done by the method described by Perrie and Perry (*23*). Protein concentration was determined by the improved method of Bradford (*24*), using bovine serum albumin as a standard.

RESULTS

Putative PP1 Inhibitor Proteins. We searched databases for sequences related to CPI-17, a phosphorylation-dependent inhibitor of protein type-1 phosphatase (PP1) that is exclusively expressed in smooth muscle. Human and mouse sequences called phospholipase Cβ neighboring gene (PNG) are open reading frames that show close similarity to CPI-17. Though the wide tissue distribution of mRNA for PNG had been studied (*17, 25*), nothing was known of the corresponding protein. No closely related sequences were found in *Drosophila*, *C. elegans*, or *S. cerevisiae* genomes. Proteins encoded by both the human and mouse PNG have significant sequence identity (29%) to the pig CPI-17 protein (Figure 1A), including a completely conserved segment R-X_3-V-T-V-K-Y-D-R containing the Thr that is phosphorylated to activate CPI-17. The alignment matched amino acid sequences between CPI-17 and only the last 3/4 of the predicted PNG proteins. We noticed that there were two possible initiation sites for translation, one at the beginning of the PNG open reading frame, and the second at a Met codon about 1/4 into the coding region. The second potential initiation site fully conformed to the Kozak concensus sequence preferred for initiation (*26, 27*), whereas the first site had C instead of G at both the −3 and +4 positions (Figure 1B). This analysis predicts that initiation would be preferred from the second Met in the PNG open reading frame, producing a 147-residue protein that more closely resembles CPI-17. Accordingly, we named the shorter and longer putative proteins encoded by the PNG as PHI-1 and PHI-2 (phosphatase holoenzyme inhibitor, see below).

Production and Purification of Recombinant Protein. We obtained a mouse embryo DNA for an EST clone of PHI-1 and inserted it into an inducible expression vector to produce a fusion protein with an N-terminal 44 residues, including a His6 tag and an enterokinase cleavage site. This protein, called H6[44]-PHI-1, was expressed in *E. coli* and purified by affinity chromatography and gel filtration (Figure 2). In addition, a mutated H6[44]-PHI-1 protein was purified, with Ala substituted for Thr57, which corresponds to the phosphorylated residue in CPI-17. The H6[44]-PHI-1 fusion protein eluted in gel filtration as a single symmetrical peak with a M_r of 46 kDa (Figure 2A), consistent with either a stable dimer, or an extended polypeptide that exhibits an anomalous size in solution. The H6[44]-PHI-1 preparation migrated in SDS-PAGE as a single band with an apparent M_r of 30 kDa (Figure 2B). Cleavage with enterokinase yielded a recombinant PHI-1 protein with a predicted mass of 16 kDa that appeared as 23 kDa in SDS-PAGE (Figure 2B, lane 5). Thus, both the fusion protein and the cleaved PHI-1 protein itself migrated less than proteins of these sizes. Rabbits were immunized with the purified fusion protein and antibodies were affinity purified with immobilized PHI-1 prepared by cleavage of the fusion protein.

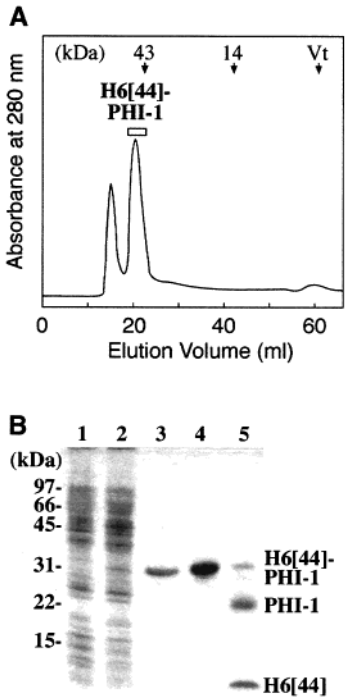

FIGURE 2: Purification of H6[44]-PHI-1 fusion protein. (A) Elution profile of H6[44]-PHI-1 from Sephadex G-75 superfine column. Ovalbumin and lysozyme were used as standards for 43 and 14 kDa, respectively. From this elution the relative molecular mass of H6[44]-PHI-1 is 46 kDa. (B) Analysis of purification by SDS-PAGE. Samples of bacteria lysate before induction (lane 1), lysate after induction with IPTG (lane 2), eluate from Ni^{2+}-NTA resin (lane 3), eluate from Sephadex G-75 gel filtration (lane 4), and enterokinase digests of H6[44]-PHI-1 (lane 5) were subject to SDS-PAGE on Phastgel 20 and proteins were stained by Coomassie blue. On the basis of migration in this gel, the relative molecular masses of H6[44]-PHI-1 and PHI-1 are 30 and 23 kDa, respectively.

Distribution of PHI-1, PHI-2, and CPI-17 in Tissues and Cell Lines. The expression of PHI-1 and PHI-2 in tissues and cell lines was examined by immunoblotting (Figure 3). Proteins corresponding to PHI-1 (23 kDa) and PHI-2 (26 kDa) were detected in multiple tissues, with a clearly different pattern of distribution. Staining of both these proteins was completely blocked by preincubating the antibodies with recombinant H6[44]-PHI-1 (not shown). PHI-1 was detected in all the tissues tested, with highest concentrations in aorta, bladder, and liver. PHI-2 was detected almost exclusively in muscles, with highest concentrations in heart, aorta, and skeletal muscle. Only PHI-1, not PHI-2, was detected in spleen, thymus, and lung. The concentration of PHI-1 was estimated as 10^{-7} M in aorta and 10^{-8} M in brain based on staining by Western blotting. The calculation assumes that the protein is present in all cells of the tissue, and 40% of the wet weight of tissues are cells. The concentration of PHI-1 in aorta is the same as that of CPI-17 in pig aorta (*13*). By comparison, immunoblotting for CPI-17 (Figure 3) showed its restricted expression in the smooth muscles of aorta, bladder, and lung, with low levels in brain and liver, presumably from arterial smooth muscles in these tissues. In tissue culture cell lines, PHI-1 was predominant over PHI-2 (Figure 3B) and CPI-17 was absent (not shown). The results show broad but differential expression of PHI-1 and PHI-2 in various tissues and cell lines.

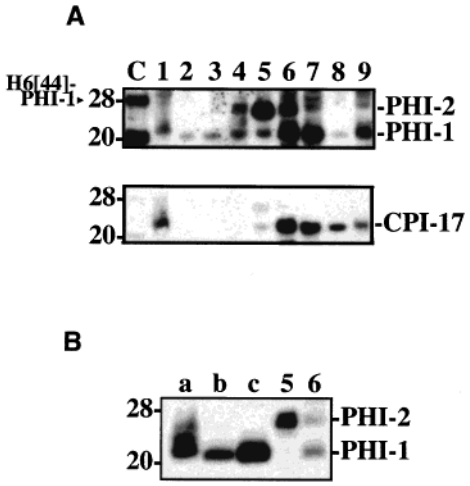

FIGURE 3: Expression of PHI-1, PHI-2, and CPI-17 in rat tissues and cultured cells. Samples of extracts from rat brain (lane 1), spleen (lane 2), thymus (lane 3), skeletal muscle (lane 4), cardiac muscle (lane 5), aorta (lane 6), bladder (lane 7), lung (lane 8), and liver (lane 9), or COS7 (lane a), NIH3T3 (lane b), and 10T1/2 (lane c) were subjected to immunoblotting using anti-PHI-1 (panel A, top frame and panel B), and anti-CPI-17 (panel A, bottom) antibodies. C denotes control lane where enterokinase-digested H6[44]-PHI-1 (5 ng) was loaded.

Phosphorylation of Wild-Type and T57A Versions of H6-[44]-PHI-1. The H6[44]-PHI-1 fusion protein was phosphorylated at two sites by purified delta isoform of protein kinase C (PKC) (Figure 4). We used PKC, because it effectively phosphorylates CPI-17 at a site that potentiates inhibitory activity, and this site is nearly identical in PHI-1. Reaction of H6[44]-PHI-1 with PKC plus radiolabeled ATP showed a time-dependent increase in ^{32}P-labeling of the protein and a coincident shift in the mobility of the protein on urea-PAGE (Figure 4A). Products were identified as singly or doubly phosphorylated by the stepwise change in electrophoretic mobility and their specific radioactivity. During the first 30 min of reaction with PKC, the major product was singly phosphorylated, and at longer reaction times there was progressive accumulation of a doubly phosphorylated product. Mutation of Thr57 to Ala eliminated one site of PKC phosphorylation in H6[44]-PHI-1 (Figure 4B). The kinetics of reaction indicated that the T57A fusion protein was only one-tenth as reactive with PKC relative to the wild-type H6-[44]-PHI-1. Even with extensive incubation only a singly phosphorylated product was formed with the T57A protein. These results make it likely that Thr57 is the first site of PKC phosphorylation in the wild-type protein. Phosphoamino acid analysis showed that wild-type H6[44]-PHI-1 was phosphorylated on both a Thr and Ser residue, whereas the T57A protein was only phosphorylated on a Ser residue (Figure 4C). The site of Ser phosphorylation has not yet been determined.

Inhibitory Properties of H6[44]-PHI-1. The protein product of the PNG open reading frame exhibited unusual properties that distinguish it from all known phosphatase inhibitor proteins, leading us to name it phosphatase holoenzyme inhibitor (PHI). The fusion protein H6[44]-PHI-1 specifically inhibited PP1, not PP2A or PP2B (Figure 5A). Unphosphorylated H6[44]-PHI-1 inhibited the PP1 catalytic subunit with an IC_{50} of 100 nM, and phosphorylation with PKC enhanced inhibitory potency 50-fold, giving an IC_{50} of 2 nM (Figure 5A). However, unlike other PP1 inhibitor

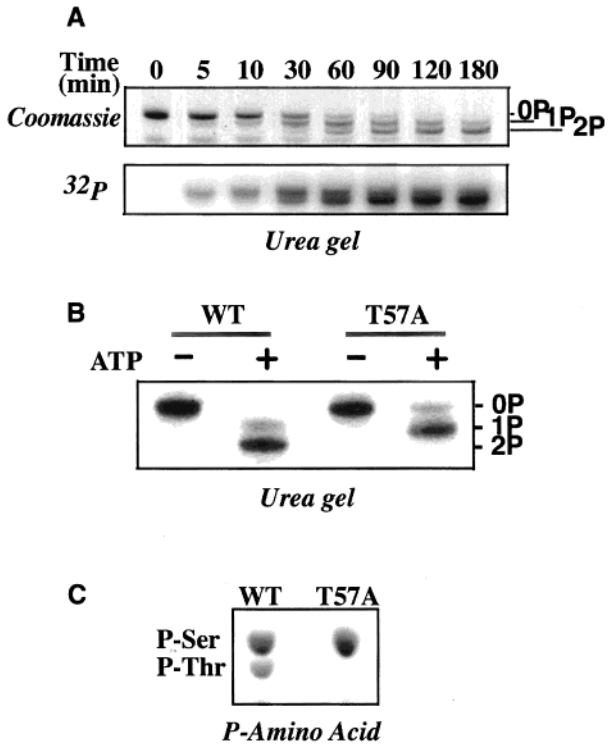

FIGURE 4: Phosphorylation of H6[44]-PHI-1 by protein kinase C. (A) Time-dependent phosphorylation monitored by Coomassie stain and autoradiography of urea gel. Phosphorylation was initiated by addition of 0.1 mM [γ-^{32}P]ATP (7.4 μCi), and at indicated time, 20 μL aliquot was taken into a tube including 10 mg of urea to terminate phosphorylation. Bands of un-, single-, and double-phosphorylated PHI-1 were indicated as 0P, 1P, and 2P, at right. (B) Phosphorylation of H6[44]-PHI-1 (WT) and T57A mutant. Recombinant proteins were phosphorylated for 120 min under the conditions used in panel A, in the presence or absence of ATP. Reaction products were analyzed by urea-PAGE and Coomassie staining. (C) Phospho-amino acid analysis of PHI-1. H6[44]-PHI-1 (WT) and T57A mutant were phosphorylated in the presence of [γ-^{32}P]ATP were partially hydrolyzed for 60 min with 6 N HCl at 110 °C. ^{32}P-labeled amino acids were subjected to electrophoresis on a cellulose-thin layer sheet according to the method of Cooper et al. (*39*).

proteins, H6[44]-PHI-1 inhibited both myosin-associated phosphatase and glycogen-bound phosphatase, using myosin light chains and phosphorylase, respectively, as substrates (Figure 5B, closed symbols). The IC_{50} of phosphorylated H6-[44]-PHI-1 for these holoenzyme forms of PP1 was 30 nM, more than an order of magnitude higher than with PP1C. Again, the phosphorylation of H6[44]-PHI-1 significantly enhanced inhibitory potency, by about 50-fold. The unphosphorylated wild-type and mono-phosphorylated (T57A)H6-[44]-PHI-1 inhibited myosin-associated phosphatase identically, in the micromolar concentration range. The results showed that phosphorylation of the Ser site in H6[44]-PHI-1 did not affect inhibitory potency in this assay, and supports our conclusion that phosphorylation of Thr57 was responsible for enhancing inhibitory activity.

DISCUSSION

Inhibition of protein Ser/Thr phosphatases is an important facet of signal transduction that has garnered significant attention recently. Phosphatases are abundant enzymes that have high catalytic activity, posing formidable opposition to signaling via protein kinases. Inhibiting phosphatase

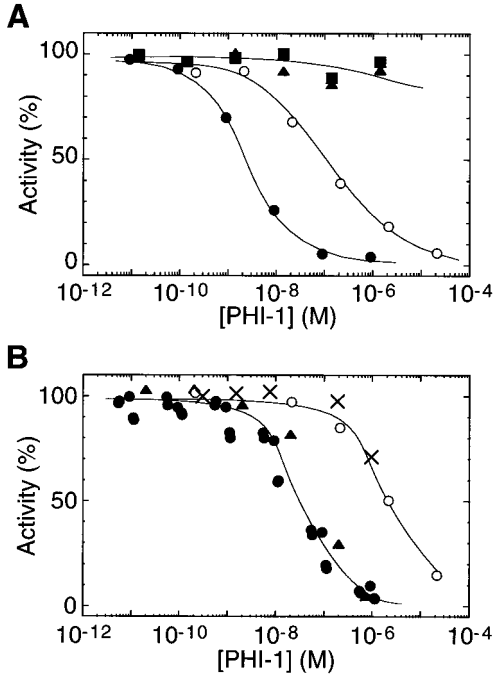

FIGURE 5: Inhibition of protein type-1 phosphatase catalytic subunit and holoenzymes by PHI-1. Phosphatase activity was assayed in the presence of phosphorylated (filled symbols and X) or unphosphorylated (open circles) H6[44]-PHI-1 at indicated concentration. (A) Inhibition of PP1C (circles), PP2A (triangle) and PP2B (square) by PHI-1. Data shown are averages from duplicate experiments. (B) Inhibition of myosin-bound PP1 (circles and X) and glycogen-bound PP1 (triangle) by PHI-1. Myosin-bound PP1 and glycogen-bound PP1 activities were measured using 1 μM ^{32}P-LC20 and 0.3 mg/mL ^{32}P-phosphorylase a as substrates, respectively. The X's indicate myosin-bound PP1 activity in the presence of singly phosphorylated H6[44]-PHI-1 (T57A) mutant. Unphosphorylated H6[44]-PHI-1 (T57A) mutant gave the same results (data not shown).

activity is an efficient means to promote and prolong signaling events within cells. Curiously, inhibition of phosphatases is produced in different ways by phosphorylation. Inhibition can be achieved by direct phosphorylation of phosphatase catalytic subunits, and this occurs in cells for PP1 and PP2A (*28*−*31*, also see (*32*)). Alternatively, regulatory subunits of PP1 and PP2A are phosphorylated to modulate activity (see refs *33*−*36*). Inhibition of myosin-associated PP1 uses this mechanism, especially involving Rho-activated kinases (*37*). Another route to inhibition of PP1 is through phosphorylation of separate inhibitor proteins. Phosphorylation-dependent inhibitors for PP1, namely, I-1 and DARPP-32, have been known and studied for years (see ref *38*)). However, these inhibitor proteins are essentially ineffective with holoenzyme forms of PP1, wherein the catalytic subunit is complexed with regulatory subunits. One exception to this is the protein CPI-17, purified from pig aorta as a phosphorylation-dependent inhibitor of myosin-associated PP1 (*11*). Phospho-CPI-17 potently inhibits myosin phosphatase activity and promotes contraction of skinned smooth muscle at submaximal [Ca^{2+}], arguing for its physiological importance (*14*). CPI-17 could be a component of Rho-activated signaling or be in a separate, parallel pathway that would converge on inhibition of myosin phosphatase and sensitization of smooth muscle contractility.

However, if CPI-17 was to represent a general mechanism for regulation of PP1, then there must be proteins close in structure and function in other tissues because CPI-17 was only detected in smooth muscle, not in other tissues or cell types. This prediction prompted us to search for proteins resembling CPI-17. We discovered related sequences from an open reading frame (ORF) that was found in the course of sequencing a genomic region implicated in endocrine tumors. This ORF was named PNG, for phospholipase Cβ neighboring gene (*25*). Northern analysis and in situ hybridization showed that the PNG mRNA was widely distributed in human and mouse tissues and was present in different regions of the brain, showing a developmental program of expression (*17*, *25*). Interestingly, the PNG sequence seems to be exclusively in vertebrates, or complex higher eucaryotes, because related sequences were not found in flies, nematodes, or yeast. This may be an example of a protein that appeared late in evolution. However, the protein encoded by PNG has not been studied and nothing was known about its function. We aligned the predicted amino acid sequence of PNG with that of CPI-17 and found 29% identity over the entire CPI-17 sequence. But, CPI-17 did not overlap at all with the 5′ end of the ORF. As we report here, there are two possible translation initiation sites, one conforming, one not, to the Kozak concensus sequence. We named these two putative proteins PHI-1 and PHI-2.

Recombinant PHI-1 was expressed and purified as a fusion protein, and its properties were explored. The fusion protein eluted as a single peak during gel filtration at double the expected size, indicating that it may form a stable dimer. Alternatively, like other PP1 inhibitor proteins, the H6[44]-PHI-1 may assume an elongated shape with high frictional coefficient and elute early from gel filtration. We do not yet have evidence to distinguish between these possibilities. The H6[44]-PHI-1 was phosphorylated on one Ser and one Thr by purified PKC. Mutation of Thr57 to Ala, which lies within a segment of sequence strictly conserved among human and mouse PHI and pig CPI-17, eliminated the Thr phosphorylation site, but this protein was still phosphorylated on one Ser. Although CPI-17 also has a Ser-phosphorylation site, the Ser phosphorylated in H6[44]-PHI-1 need not be same. Regardless, there are only a few possibilities for the phosphorylated Ser in PHI, and we speculate that because of the basic residues at −6, −2, and +4 it may be the C-terminal K-L-**S**-T-P-Q-K-K. Using antibody affinity purified with the recombinant PHI-1, proteins of sizes matching both PHI-1 and PHI-2 were detected in various tissues. The PHI-1 was expressed to varying levels in all tissues and all cell lines tested, and therefore, it may be the widely distributed phosphoinhibitor orthologue of CPI-17 we were seeking. PHI-2 also was intriguing because its expression was restricted to various muscles and was especially prominent in heart, implying a possible role in control of cardiac function through inhibition of PP1.

Most important, our results reveal the novel functional activity of H6[44]-PHI-1. Like CPI-17, the PHI-1 fusion protein was a specific inhibitor of PP1C compared to PP2A and PP2B. Like other PP1 inhibitor proteins such as I-1, DARPP-32, and CPI-17, phosphorylation of one site in the inhibitor greatly enhanced inhibitory potency. For the other inhibitors, enhancement is 1000- to 2000-fold, converting proteins with micromolar binding affinity into inhibitors with nanomolar potency. With the PHI-1 fusion protein, there was only a 50-fold increase in the inhibitory potency. It is at least

possible that the PHI proteins may bind and/or inhibit PP1 in cells even without being phosphorylated. In this scenario, phosphorylation of a bound PHI might serve to further reduce phosphatase activity. However, because the concentration of PHI-1 in muscle was estimated as 10^{-7} M, it is likely that inhibition of PP1 by PHI-1 is controlled by its phosphorylation. This would establish a novel link between activation of PKC and inhibition of PP1. Unlike any other PP1 inhibitor protein, including CPI-17, the PHI-1 fusion protein inhibited the phosphorylase phosphatase activity of glycogen-bound PP1. This implies a new mode of inhibition that obviates interference by bound regulatory subunits. Though CPI-17 can inhibit PP1C bound to the myosin binding subunit, it is not able to inhibit glycogen-bound PP1.[3] Therefore, the MYPT subunit and G subunits (G_M or PTG) must occlude different regions of the PP1C surface, one allowing access for CPI-17, the other preventing access. At the very least the MYPT subunit must use contact sites on PP1C distinct from those used by CPI-17, which inhibits the holoenzyme containing MYPT. Now, the PHI proteins present yet another variation, because neither the myosin-binding or glycogen-binding subunits of PP1 prevent inhibition by PHI. All the more curious, both PHI-1 and PHI-2 have a R-V-Y-F sequence (see Figure 1) that is thought to be a motif common to all PP1C binding proteins. If indeed this motif mediates binding to PP1C, then PHI and regulatory subunits would need to use the same site, without mutual exclusion or dissociation. Alternatively, other motifs in PHI might be used to interact with PP1C at sites not yet recognized. This would require rethinking the prevailing R-V-X-F paradigm for PP1 subunit association. Regardless, the PHI proteins present us with new possibilities to link phosphorylation pathways to inhibition of PP1 holoenzymes in cells. While increasing complexity by increasing the number and types of signaling components, this new information may well lead us to a more detailed and comprehensive model for intracellular networks that respond to multiple, simultaneous signals.

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