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Regulation of Cellular Protein Phosphatase-1 (PP1) by Phosphorylation of the CPI-17 Family, C-kinase-activated PP1 Inhibitors*[S]

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The regulatory circuit controlling cellular protein phosphatase-1 (PP1), an abundant group of Ser/Thr phosphatases, involves phosphorylation of PP1-specific inhibitor proteins. Malfunctions of these inhibitor proteins have been linked to a variety of diseases, including cardiovascular disease and cancer. Upon phosphorylation at Thr^{38}, the 17-kDa PP1 inhibitor protein, CPI-17, selectively inhibits a specific form of PP1, myosin light chain phosphatase, which transduces multiple kinase signals into the phosphorylation of myosin II and other proteins. Here, the mechanisms underlying PP1 inhibition and the kinase/PP1 cross-talk mediated by CPI-17 and its related proteins, PHI, KEPI, and GBPI, are discussed.

The reciprocal activities of protein kinases and phosphatases determine protein phosphorylation levels in cells. PP1[2] dephosphorylates phospho-Ser/Thr residues of proteins to regulate multiple signaling pathways at various cellular loci (1–3). Cellular PP1 is associated with PP1 regulatory proteins/subunits at their PP1-binding site, known as the RV*X*F motif. The binding of PP1 regulatory proteins thus confers substrate specificity and localization on cellular PP1. Nearly 100 polypeptides have been identified as PP1 regulatory proteins, and these account for the wide spectrum of PP1 function (1–3). In addition, eukaryotic cells express several PP1 inhibitor proteins that play important roles in regulating cellular PP1. The first generation of PP1 inhibitor proteins involves inhibitor-1, inhibitor-2, DARPP32, and NIPP-1, which potently inhibit the free catalytic subunit of PP1, but these inhibitor proteins were much less potent toward purified PP1 holoenzymes, MLCP and glycogen-bound PP1. Therefore, cellular PP1 holoenzymes were thought to undergo subunit dissociation prior to the inhibition of PP1 by the inhibitor proteins (1, 2). However, the number of PP1 holoenzymes that do undergo subunit dissociation in cells remains unclear.

MLCP is a trimeric PP1 holoenzyme, consisting of a PP1∂ isoform and a regulatory complex of MYPT1 (aka MBS, M110) and a 21-kDa accessory subunit, and vital to control cellular phosphorylation in response to various signals (4). MYPT1 and PP1 bind through the MYPT1 KVKF segment, as well as its eight-repeat ankyrin motif at the N-terminal domain (5). Binding of the N-terminal 300-residue domain of MYPT1 is sufficient to allosterically regulate PP1 activity. The MYPT1 C-terminal domain directly binds to substrates, including myosin and ezrin/radixin/moesin (4). MLCP activity is reversibly regulated in response to various signals. For example, in smooth muscle, activation of the G-protein-coupled receptor inhibits MLCP, resulting in increased Ca^{2+} sensitivity of myosin phosphorylation and contraction, whereas cyclic nucleotide signals can activate MLCP to induce smooth muscle relaxation (6). MLCP inhibition occurs upon MYPT1 phosphorylation at Thr^{696} and Thr^{853} (4). On the other hand, protein kinase G can activate MLCP (7). These regulatory signals are MYPT1 isoform-dependent (8), suggesting an important role for MYPT1 in MLCP regulation. In addition, we identified the MLCP inhibitor protein, named CPI-17, which transduces G-protein signals into MLCP inhibition (9, 10). Based on sequence similarity, three CPI-17 homologs in the human genome, PHI, KEPI, and GBPI, were characterized as PP1 inhibitors (11–13). Each CPI-17 family member carries a PHIN domain, in which the sequences are >41% identical to CPI-17 (Fig. 1*A*). Indeed, all CPI-17 family members potently inhibit MLCP activity, which suggests new avenues for PP1 holoenzyme inhibition. This minireview will focus on CPI-17 and its homologs (whose amino acid sequences differ significantly from other PP1 inhibitor proteins), highlight critical findings from CPI-17 studies, and discuss the role of other CPI-17 family members in regulating PP1 activity.

Structure and Function of CPI-17

Amino Acid Sequence of CPI-17—The CPI-17 gene (*PPP1R14A*, chromosome 19) encodes a 147-residue polypeptide in which >85% of the amino acids are identical within mammals (10) (Fig. 1*A*). A splice variant of CPI-17 (CPI-17β) lacking exon 2 exists in human smooth muscle cells, although whether this form is physiologically relevant is not known (see below) (14). Zebrafish express a similar gene, although to which CPI-17 family member this gene product is functionally related is unclear. No homologous genes have been detected in fruit fly, nematode, and yeast, suggesting that the CPI-17 family emerged at a late stage in evolution. Phosphorylation of CPI-17 at Thr^{38} is necessary and sufficient to convert the protein into a potent MLCP inhibitor (9, 10). No homology is detected between the CPI-17 family and other classes of PP1 inhibitors, such as inhibitor-1 and inhibitor-2, even though phosphoryla-

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[2] The abbreviations used are: PP1, Ser/Thr protein phosphatase-1; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase-targeting subunit-1; CPI-17, C-kinase-activated PP1 inhibitor of 17 kDa; PHI, PP1 holoenzyme inhibitor; KEPI, kinase C-enhanced PP1 inhibitor; GBPI, gastrointestinal- and brain-specific PP1 inhibitor; PHIN, PP1 holoenzyme inhibitory; PKC, protein kinase C; ROCK, RhoA-activated coiled-coil kinase; ILK, integrin-linked kinase; PKA, protein kinase A; ERK1/2, extracellular signal-regulated kinase-1/2; PNG, phospholipase C-neighboring gene; I-2, inhibitor-2.

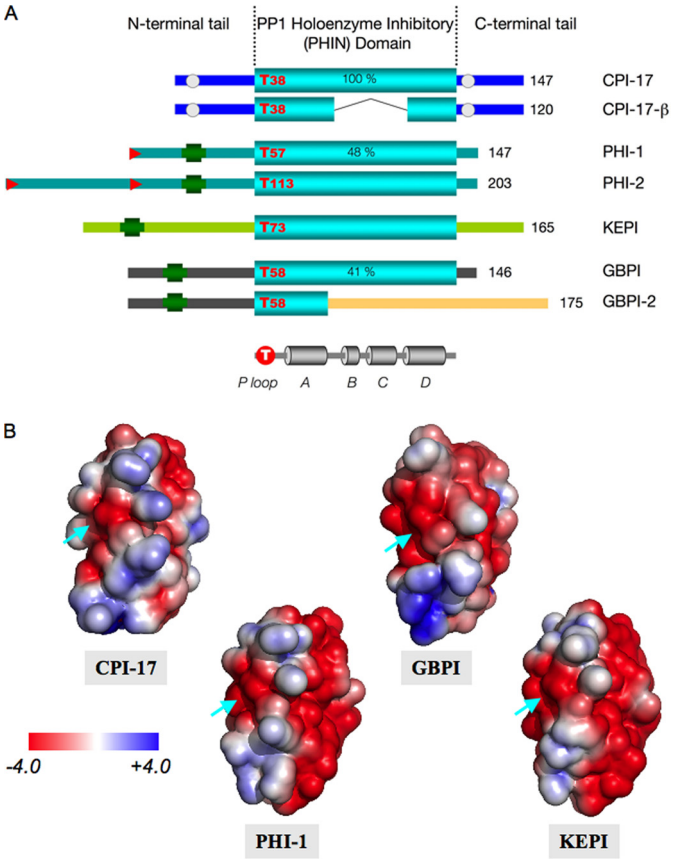

FIGURE 1. **CPI-17 family.** *A*, schematic illustration of the CPI-17 family primary structure. The inhibitory phosphorylation site (*red*) is located in the conserved PHIN domain (*cyan boxes*). *Light gray dots* and *green boxes* indicate additional phosphorylation sites and PP1-binding motifs, respectively. *B*, electrostatic surface potential map of the CPI-17 family. The surface model of phospho-CPI-17 was used as a template, and putative models for other proteins were generated *in silico* based on the sequence alignment. The surface modeling was performed by Altif Laboratories (Tokyo, Japan).

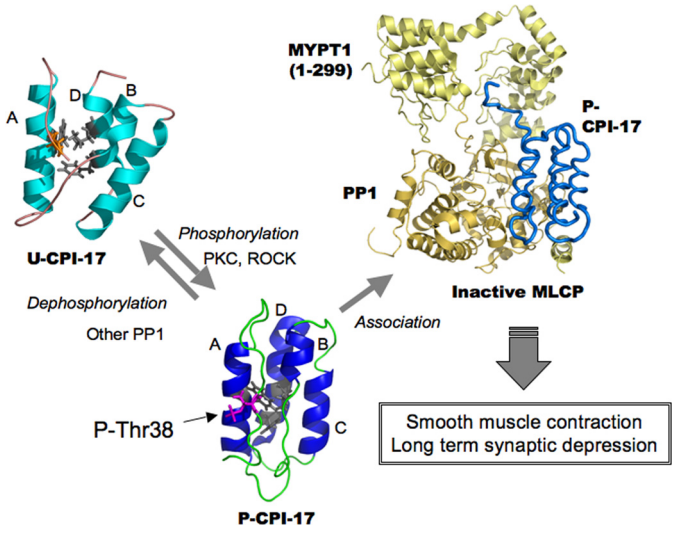

FIGURE 2. **Model for selective inhibition of MLCP by phospho-CPI-17.** Upon phosphorylation of Thr^{38}, CPI-17 undergoes a conformational change that results in a realignment of the four helices, A–D (*middle*). Phospho-CPI-17 docks at the active site of MLCP and suppresses its activity (*right*). Other PP1 holoenzymes can dephosphorylate phospho-CPI-17 and neutralize its inhibitory potency. *U-CPI-17* and *P-CPI-17*, unphosphorylated and phosphorylated CPI-17, respectively.

tion is also involved in the function of most other PP1 inhibitor proteins. The CPI-17 structure has three domains: N- and C-terminal tails and the central 86-residue PHIN domain between residues 35 to 120 (Fig. 1*A*) (15). The sequence surrounding the inhibitory phosphorylation site characterizes the CPI-17 family and is pseudo-palindromic, (basic)-(hydrophobic)-Thr-(hydrophobic)-(basic) (16). Tyr^{41}, Asp^{42}, and Arg^{43} of CPI-17 are necessary for the inhibitory activity and are also conserved among CPI-17 family members (15). Substitution of Ala at CPI-17 Tyr^{41} accelerates phospho-Thr^{38} dephosphorylation, the significance of which will be discussed (15). In contrast to the PHIN domain, both the N- and C-terminal tail domains are unique for each CPI-17 family member. The other CPI-17 family members do possess the putative PP1-binding RV*X*F motif, which is located at the N-terminal tails of PHI, KEPI, and GBPI (Fig. 1*A*, *green boxes*) (11–13).

Three-dimensional Structure of CPI-17—Solution NMR studies revealed the three-dimensional structure of unphosphorylated and phospho-CPI-17 PHIN domains (16). The structure of the CPI-17 PHIN domain consists of a loop structure encompassing the phosphorylation site Thr^{38} (P-loop), followed by a four-helix bundle that stabilizes the P-loop structure (Fig. 1*A*, *lower*) (16). Fig. 2 shows the three-dimensional structural models of unphosphorylated and phospho-CPI-17. In the unphosphorylated form, two paired A/D- and B/C-helices form a V-shaped structure with the P-loop situated between the paired helices (Fig. 2, *left*). Upon Thr^{38} phosphorylation, the P-loop becomes more solvent-exposed and, in doing so, generates torque in the A-helix. This twisting of the A-helix rolls the A-B-loop up to align the B/C-helices in parallel with the A/D-helices (Fig. 2, *center*). The newly aligned four helices are then stabilized through a hydrophobic core that is created by the rearrangement. The P-loop of phospho-CPI-17 is now displayed on the molecular surface, tethered by Tyr^{41}. Presumably, the anchoring function of Tyr^{41} is necessary to prevent dephosphorylation of the MLCP active site. The phosphate group at Thr^{38} cannot be replaced with Asp, which causes P-loop dislocation with a minimal increase in inhibitory potency, or with Glu, which distorts the overall structure. Furthermore, substitution of a cysteine-derived sulfonic acid side chain at Thr^{38} cannot mimic phosphorylation. Thus, the phosphate group seems to play a specific role in the potent inhibitory activity of CPI-17 beyond being only a trigger of conformational change. The splice variant CPI-17β retains the P-loop and the A/D-helix pair, although whether this isoform can inhibit PP1 or functions as a dominant-negative form in the cell is not known. Based on the sequence similarity in the PHIN domain, the structural topology, and as such the function, is likely conserved for CPI-17 family members.

Selective Inhibition of MLCP by Phospho-CPI-17—Phospho-CPI-17 selectively inhibits the MLCP complex with an IC_{50} value of ~1 nM (17, 18). How then can CPI-17 recognize only the PP1 associated with MYPT1 among nearly 100 other PP1 holoenzymes that exist in cells? Fig. 2 illustrates our current model for the selective inhibition of MLCP by phospho-CPI-17. PP1 associated with MYPT1 is unable to hydrolyze phospho-Thr^{38} of CPI-17, so phospho-CPI-17 forms a stable complex with MLCP (Fig. 2, *left*). On the other hand, other PP1 holoen-

zymes are able to dephosphorylate phospho-CPI-17 and neutralize its inhibitory potency. Simply put, the PP1 regulatory subunit determines whether phospho-CPI-17 is an inhibitor or substrate of PP1. A kinetic analysis suggests that a mixed inhibition of MLCP is induced by phospho-CPI-17, with K_i and K_i' values of 1.9 and 5.1 nM, respectively (17). Indeed, computer modeling predicts a direct contact between phospho-CPI-17 and MYPT1 (Fig. 2, *right*), which may account for the specific inhibition of MLCP by CPI-17 (16). Fig. 1*B* illustrates the electrostatic surface potential of phospho-CPI-17 and the predicted maps for other CPI-17 homologs calculated from sequence alignments. The docking surface of CPI-17 (Fig. 1*B*, *left*) consists of positively charged residues surrounding an acidic island of phospho-Thr^{38} (*cyan arrow*). The positively charged regions around phospho-Thr^{38} seem to complement the acidic cluster formed by PP1 and the MYPT1 ankyrin repeat domain (5). The pattern of surface potential varies within the CPI-17 family, whereas the negative charge dominates in models of PHI-1 and KEPI structures, and the positive charge is clustered at the edge of GBPI (Fig. 1*B*). The differences in the structure of the docking surface suggest that each CPI-17 homolog selectively controls a specific subset of target PP1 holoenzymes and cellular events.

Role of CPI-17 in Cell Signaling

Kinases and Phosphatases Regulating CPI-17—Multiple kinases and phosphatases are involved in regulating CPI-17 phosphorylation. In smooth muscle, CPI-17 phosphorylation occurs in response to agonist stimulation through activation of PKC, ROCK, and ILK (19, 20). Indeed, PKCα and PKCδ are the dominant kinases for CPI-17 in pig aorta smooth muscle extracts (21). Also, CPI-17 binds to the regulatory domain of PKC isoforms, including α, ϵ, λ, ζ, and μ (22). Zipper-interacting kinase and p21-activated kinase are also known to directly phosphorylate isolated CPI-17 at Thr^{38} (23, 24). Thus, CPI-17 is expected to function as a hub of multiple kinase signals that control MLCP activity. For example, α_1-adrenergic receptor stimulation produces biphasic phosphorylation of CPI-17 through the sequential activation of PKC and ROCK in smooth muscle (supplemental Fig. 1) (25). The G-protein-coupled receptor-induced rapid activation of Ca^{2+}-dependent PKC elicits acute CPI-17 phosphorylation, causing MLCP inhibition, which amplifies the Ca^{2+}/calmodulin-dependent myosin light chain kinase signal. Following Ca^{2+} withdrawal, the delayed and sustained activation of ROCK maintains CPI-17 and MYPT1 phosphorylation, causing tonic smooth muscle contraction (25). Thus, the combination of kinase signals confers the profile of smooth muscle force generation through CPI-17 phosphorylation. CPI-17 phosphorylation reversibly declines in response to elevated cAMP/cGMP levels (26), which attenuate PKC and ROCK signals (27). In addition, treatment with a cGMP analog possibly activates unidentified phosphatase(s) that can dephosphorylate CPI-17 (28). In our model, CPI-17 is dephosphorylated by such "other" PP1 complexes (Fig. 2) (18). In addition, purified PP2A and PP2C are capable of dephosphorylating CPI-17 (29), suggesting the possible involvement of multiple phosphatases in regulating CPI-17 phosphorylation. Interestingly, PKA is known to phosphorylate and activate PP2A in brain (30), so CPI-17 dephosphorylation could occur through cAMP/cGMP-activated PP2A. It should be noted that high activity of CPI-17 phosphatase(s) may explain why CPI-17 phosphorylation cannot be detected in thromboxane A_2-stimulated cerebral artery from normal rat (31) or phenylephrine-stimulated mesentery arteries from genetically hypertensive rats (32). In addition to Thr^{38}, purified PKC also phosphorylates Ser^{12} at the CPI-17 N-terminal tail, whereas Ca^{2+}/calmodulin-dependent protein kinase II preferentially phosphorylates Ser^{130} at the CPI-17 C-terminal tail (9, 33). CPI-17 phosphorylation at Ser^{128} was also detected in brain tissue extracts (33). However, the physiological relevance of this additional phosphorylation at both tails remains to be investigated. The possibility that these sites are involved in regulating other target subsets, as reported for the phosphorylation of DARPP32 at Thr^{34} and Thr^{75}, which induces the inhibition of PP1 and PKA, respectively (34), cannot be discounted, however.

Expression of CPI-17—CPI-17 is expressed predominantly in mature smooth muscle (10), and higher levels are present in tonic muscles, such as arteries (at 7 μM), compared with phasic muscles, such as ileum, bladder, and vas deferens (at 0.8 μM), or cells in neointimal lesions (35, 36). CPI-17 is also expressed in embryonic cardiac muscle, in which smooth muscle marker proteins are expressed, but its expression disappears in adult tissue (36). Platelets, neurons, endothelia, and epithelia also express CPI-17, whose roles in these tissues will be discussed (35–37). Accumulating evidence suggests a correlation between the CPI-17 expression level and the extent of PKC-mediated Ca^{2+}-sensitized force. Selective permeabilization of smooth muscle tissue with Triton X-100 eliminates the contraction induced by PKC activation, and the addition of recombinant CPI-17 restores PKC-mediated contraction (38). The extent of smooth muscle contraction evoked by phorbol ester stimulation depends on the CPI-17 expression level (35). Interestingly, CPI-17 is absent in tissues from the American farm chicken and as such provides an excellent model of CPI-17-null smooth muscle (39). Stimulation with agonists, phorbol 12,13-dibutyrate, or G-proteins evokes a marginal extent of the contraction of chicken aortic smooth muscle, suggesting the importance of CPI-17 in agonist-induced smooth muscle contraction (39). Furthermore, fluctuations in CPI-17 signals reportedly occur under pathological conditions, such as hypertension, asthma, inflammation, and diabetes (40–45). For example, CPI-17 expression and phosphorylation are up-regulated in hypoxia-induced pulmonary hypertension (40). CPI-17 up-regulation is also found in airway smooth muscle during inflammation and in diabetic bladder smooth muscle (41, 45). In contrast, inflammation causes down-regulation of CPI-17 in intestinal smooth muscle in parallel with a reduction in muscle tone (43). How inflammatory signals trigger this bidirectional regulation of CPI-17 in different smooth muscle tissues remains unknown.

CPI-17 in Other Cell Types—Reversible phosphorylation of myosin is involved in controlling endothelial cell motility and platelet activation. CPI-17 in endothelial cells and platelets translates the activation of PKC and/or ROCK into MLCP inhibition and myosin II phosphorylation as seen in smooth muscle

(46, 47). In Purkinje neurons, CPI-17 is involved in long-term synaptic depression (37). The synaptic depression of cerebellar Purkinje cells occurs through PKC-mediated chronic internalization of the AMPA receptor in response to glutamate release. Neutralization of endogenous CPI-17 in Purkinje cells using small interfering RNA or a blocking antibody results in rapid recovery of membrane current upon glutamate stimulation (37), suggesting that metabotropic Glu receptor-induced activation of PKC causes CPI-17 phosphorylation and subsequent MLCP inhibition, thus maintaining AMPA receptor internalization (37). Furthermore, CPI-17 drives cell proliferation by activating the mitogen-activated protein kinase signaling pathway (48). Growth factor signals induce phosphorylation of merlin, a product of the neurofibromatosis type 2 gene, which relieves inhibition of the ERK1/2 signal. Merlin is phosphorylated by a subset of protein kinases, including ROCK, p21-activated kinase, and PKC, which are also capable of phosphorylating CPI-17. Overexpression of CPI-17 down- and up-regulates MLCP and merlin phosphorylation, respectively, and attenuates the tumor suppression activity of merlin (48, 49). Over 90% of cancer cells are derived from epithelial cells, in which trace amounts of CPI-17 are expressed (35, 36). The role of CPI-17 in normal epithelium remains to be investigated.

Functions of Other CPI-17 Family Members

PHI—Both PHI-1 and PHI-2 are products of PNG (*PPP1R14B*) (50). PNG was originally discovered on chromosome 11 as a candidate gene involved in multiple endocrine neoplasia type 1, although later studies eliminated that possibility. Two potential initiation ATG sequences exist in the PNG transcript (11, 50). Initiation at the first ATG yields a 203-residue polypeptide, named PHI-2, whereas the other in-frame ATG initiates translation for the 147-residue polypeptide, PHI-1 (Fig. 1*A*, *red triangles*) (11). PHI-1 is ubiquitously and abundantly expressed in various tissues and cultured cells. In contrast, PHI-2 expression is restricted to muscle tissues (11). Immunohistochemical analysis showed a significant difference between CPI-17 and PHI-1/2 localization, with the antibody recognizing both PHI-1 and PHI-2 heavily staining skeletal muscle capillary endothelium and the juxtamembrane region of the ileac smooth muscle layer (51). Recombinant PHI-1 inhibits PP1 and the purified MLCP complex upon phosphorylation at Thr^{57} (11). Phosphorylated PHI-1 evokes contraction of skinned smooth muscle strips (52). However, the inhibitory potency of PHI-1 for the MLCP complex (IC_{50} = 50 nM) is significantly lower compared with that of CPI-17 (IC_{50} = 1 nM), suggesting novel target PP1 holoenzymes for PHI-1. Purified PKC and ROCK are capable of phosphorylating PHI-1 at Thr^{57} and other undetermined site(s) (52), whereas ILK phosphorylates PHI-1 exclusively at Thr^{57} (52). Activation of G-proteins in smooth muscle tissues induces the phosphorylation of endogenous PHI-1 (53, 54). On the other hand, reconstitution of unphosphorylated PHI-1 does not restore phorbol ester-induced contraction of CPI-17-null chicken smooth muscle (39). Therefore, PHI-1 is not involved in PKC-mediated MLCP inhibition. The endothelial expression of PHI-1 is involved in cell migration (55). Endogenous PHI-1 accumulates at the leading edge of endothelial cells, and gene silencing of PHI-1 can retard cell migration. Interestingly, PHI-1 knockdown does not affect the phosphorylation status of MLCP substrate proteins, such as myosin light chain and ezrin/radixin/moesin (55), suggesting that PHI-1 controls a novel subset of PP1 holoenzymes in endothelial cells.

KEPI and GBPI—KEPI (*PPP1R14C*, chromosome 6) was discovered as a protein that is up-regulated in brain tissue isolated from morphine-addicted mice (12). In terms of amino acid sequence, KEPI seems to be more closely related to PHI-1 than CPI-17. The phosphorylation of KEPI at Thr^{75} by PKC is sufficient to convert this protein into a potent PP1 inhibitor (12). A PP1-binding motif (-KVFF-) exists in the N-terminal tail of KEPI (Fig. 1*A*, *green boxes*). Indeed, PP1 coprecipitates with beads conjugated to unphosphorylated KEPI (56). Purified PKC and ILK phosphorylate recombinant KEPI at Thr^{73}, the inhibitory phosphorylation site (12, 57). Phospho-KEPI inhibits the purified MLCP complex and isolated PP1 with IC_{50} values of 8 and 0.1 nM, respectively (57). Therefore, phospho-KEPI potently inhibits the PP1 holoenzyme, but the N-terminal KVFF sequence of KEPI may affect its inhibitory potency. Recently, KEPI was rediscovered in a group of genes that are down-regulated in breast tumor cells, along with a known tumor suppressor, EGR1 (early growth response gene-1) (58). Ectopic expression of KEPI in MCF-7 cells restores the expression of EGR1 and its downstream proteins, such as PTEN (phosphatase tensin homolog). Although both CPI-17 and KEPI are involved in cell growth regulation, there is a clear contrast in their downstream signals, suggesting that different pools of target PP1 holoenzymes exist for each inhibitor (48, 58). GBPI (*PPP1R14D*, chromosome 15) was discovered as a homolog of KEPI (13). The gene transcribes two splicing variants, GBPI and GBPI-2 (Fig. 1*A*). GBPI includes an intact PHIN domain whose sequence is 35% identical to CPI-17. On the other hand, the testis-specific GBPI-2 mRNA includes a frameshift at the A-B-loop and as such is unlikely to inhibit PP1. GBPI phosphorylated by PKC inhibits isolated PP1 with an IC_{50} value of 3 nM. Phosphorylation of GBPI with PKA eliminates its inhibitory potency. A PP1-binding motif, KVHW, is found in the N-terminal tail (Fig. 1*A*, *green boxes*), which is necessary for the inhibition of the isolated PP1 catalytic subunit (13). Whether GBPI is capable of inhibiting PP1 holoenzymes has yet to be tested. Interestingly, GBPI enhances PP2A activity following its phosphorylation by PKC (13).

Cellular Regulation of PP1 Holoenzymes via PP1 Inhibitor Proteins

After the discovery of CPI-17 and CPI-17 family members, it becomes clear that PP1 inhibitor proteins characterized previously also engage in the control of cellular PP1 holoenzymes in the absence of subunit dissociation. For example, PP1 I-2 inhibits a complex of PP1 and a microtubule-binding kinase, Nek2, through the conserved C-terminal domain of I-2 (59), which directly docks at the active site of PP1 in the co-crystal model of the PP1·I-2 complex (60). In addition, PP1 inhibitor-1 and inhibitor-3 also inhibit PP1 holoenzymes (reviewed in Ref. 3). Thus, each PP1 inhibitor protein may target a specific subset of PP1 holoenzymes, and there are more PP1 inhibitor proteins that transduce kinase signals into phosphatases as >100 polypeptides function as PP1

regulatory subunits. A proteomic approach ("PP1 inhibitome") will be useful to gain a full understanding of the specific combinations of kinases, PP1 holoenzymes, and inhibitor proteins (supplemental Fig. 2). As discussed here, CPI-17, as well as other PP1 inhibitor proteins, plays vital roles in signal transduction, controlling both amplitude and duration of phosphorylation. Additional PP1 inhibitor proteins will surely be rediscovered as disease-causing genes and recognized as novel therapeutic targets.

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