The Phosphatase PHLPP Controls the Cellular Levels of Protein Kinase C*

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The life cycle of protein kinase C (PKC) is controlled by multiple phosphorylation and dephosphorylation steps. The maturation of PKC requires three ordered phosphorylations, one at the activation loop and two at COOH-terminal sites, the turn motif and the hydrophobic motif, to yield a stable and signaling-competent enzyme. Dephosphorylation of the enzyme leads to protein degradation. We have recently discovered a novel family of protein phosphatases named PH domain leucine-rich repeat protein phosphatase (PHLPP) whose members terminate Akt signaling by dephosphorylating the hydrophobic motif on Akt. Here we show that the two PHLPP isoforms, PHLPP1 and PHLPP2, also dephosphorylate the hydrophobic motif on PKC βII, an event that shunts PKC to the detergent-insoluble fraction, effectively terminating its life cycle. Deletion mutagenesis reveals that the PH domain is necessary for the effective dephosphorylation of PKC βII by PHLPP in cells, whereas the PDZ-binding motif, required for Akt regulation, is dispensable. The phorbol ester-mediated dephosphorylation of the hydrophobic site, but not the turn motif or activation loop, is insensitive to okadaic acid, consistent with PHLPP, a PP2C family member, controlling the hydrophobic site. In addition, knockdown of PHLPP expression reduces the rate of phorbol ester-triggered dephosphorylation of the hydrophobic motif, but not turn motif, of PKC α. Last, we show that depletion of PHLPP in colon cancer and normal breast epithelial cells results in an increase in conventional and novel PKC levels. These data reveal that PHLPP controls the cellular levels of PKC by specifically dephosphorylating the hydrophobic motif, thus destabilizing the enzyme and promoting its degradation.

Protein phosphorylation defines one of the most important and pervasive regulatory mechanisms in cell signaling. Crucial cellular decisions such as those between death or survival and proliferation or differentiation are made depending on the phosphorylation state of signaling molecules. Thus, precise control of the balance between phosphorylation and dephosphorylation is critical for living organisms to maintain normal physiological functions. Dysregulation of signaling pathways that results in disturbing this balance leads to the development of diseases such as cancer and diabetes. Loss of control of either phosphorylation or dephosphorylation mechanisms leads to pathogenic states, and both kinases and phosphatases have been identified as oncogenes or tumor suppressors (1).

We have recently identified a novel family of Ser/Thr phosphatases, which we named PH domain leucine-rich repeat protein phosphatase (PHLPP)[3] based on domain composition (2, 3). PHLPP comprises three isozymes: the alternatively spliced PHLPP1α and PHLPP1β, which differ in an amino-terminal extension on PHLPP1β, and PHLPP2. PHLPP1 and PHLPP2 share 50% overall identity at the amino acid level. PHLPP1 and PHLPP2 contain a PP2C-like phosphatase domain and they dephosphorylate Akt *in vitro* in a Mn^{2+}-dependent manner (2, 3). Although both PHLPP isoforms specifically dephosphorylate the hydrophobic motif of Akt (Ser[473] in Akt1) and inactivate the enzyme in cells, they differentially terminate Akt signaling by regulating distinct Akt isoforms (3). Moreover, the PHLPP-mediated termination of Akt signaling depends on the presence of a targeting module, the PDZ-binding motif, at the extreme COOH terminus of PHLPP. Given the high conservation in the phosphorylation mechanisms of Akt and PKC (4), including conservation of the hydrophobic phosphorylation motif, PHLPP is a likely candidate to regulate PKC family members.

Akt and PKC are activated by sequential phosphorylation steps at two sites conserved within the AGC kinase family (4). First, PDK-1 phosphorylates a segment at the entrance to the active site termed the activation loop (5, 6). The phosphorylation by PDK-1 triggers the phosphorylation of a site at the COOH terminus referred to as the hydrophobic phosphorylation motif and corresponds to Ser[473] in Akt1 and Ser[660] in PKC βII. For conventional PKCs (*e.g.* PKC βII), the mechanism of phosphorylation of the hydrophobic site is by intramolecular autophosphorylation (7, 8). The carboxyl terminus of Akt and PKC contains a second site, the turn motif, whose phosphorylation is required for subsequent phosphorylation on the hydrophobic motif of PKCs (4, 9, 10). The two phosphorylation

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[3] The abbreviations used are: PHLPP, PH domain leucine-rich repeat protein phosphatase; GST, glutathione S-transferase; PDBu, phorbol 12,13-dibutyrate; PDK-1, phosphoinositide-dependent kinase 1; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; siRNA, small interfering RNA; HA, hemagglutinin; mAb, monoclonal antibody; PH, pleckstrin homology; LRR, leucine rich repeat; OA, okadaic acid; WT, wild type.

switches (activation loop and COOH terminus) are conserved among AGC kinases.

Phosphorylation of the hydrophobic site regulates both Akt and PKC, but phosphate at this site has different roles in the function of these two kinases. For Akt, phosphorylation regulates the acute activity of the enzyme. Specifically, Akt phosphorylated at only the PDK-1 site has 10% of the activity of enzyme phosphorylated at both the PDK-1 site and the hydrophobic site, Ser^{473} (11). Thus, selective dephosphorylation of Ser^{473} results in effective signal termination. For PKC, phosphorylation at the hydrophobic site controls the stability of the enzyme (9, 12). Conditions that promote the dephosphorylation of this site promote the degradation of PKC.

The phosphorylation of conventional PKCs is constitutive, serving to "prime" PKC into a catalytically competent state. The enzyme is activated following generation of second messengers, which recruit PKC to the membrane, thus releasing an autoinhibitory pseudosubstrate sequence from the substrate-binding cavity. Activated PKC adopts an open conformation in which the enzyme is sensitive to dephosphorylation, triggering one pathway of down-regulation. Specifically, prolonged activation of PKC, notably following treatment of cells with tumor promoting phorbol esters, triggers dephosphorylation and subsequent association of the dephosphorylated species with the detergent-insoluble fraction of cells, leading to eventual degradation (13, 14). Note that dephosphorylation is not required for degradation; a second pathway has recently been described in which phosphorylated PKC is ubiquitinated and degraded (15).

Because dephosphorylated PKC is removed from the pool of functional PKC, the duration and amplitude of PKC signaling is critically controlled by the dephosphorylation process (14, 16). However, little is known about the phosphatases responsible for dephosphorylating PKC. It has been shown that PP2A and PP1 dephosphorylate PKC α and βII *in vitro* (16, 17). Co-localization of PP2A with PKC α was observed in the membrane fraction of phorbol ester-treated cells (18). In addition, pretreatment of cells with okadaic acid, an inhibitor of PP2A and PP1 phosphatases, inhibits phorbol ester-induced dephosphorylation of PKC α (18).

This study addresses the role of PHLPP in dephosphorylating conventional PKC. We show that overexpression of either PHLPP1 or PHLPP2 in cells results in accumulation of dephosphorylated PKC βII in the detergent-insoluble fraction of cells. Conversely, depletion of PHLPP1 or PHLPP2 using RNA interference inhibits the phorbol ester-induced dephosphorylation of endogenous PKC α specifically at the hydrophobic motif site. Furthermore, knockdown of both PHLPP isoforms in cancer and normal cells results in an increase in PKC expression. Thus, PHLPP specifically dephosphorylates the hydrophobic motif of PKCs, effectively terminating the lifetime of PKC.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—Okadaic acid, PDBu, PMA, and MG-132 were purchased from EMD/CalBiochem. PHLPP1- and -2-specific SmartPool siRNAs were obtained from Dharmacon. The following antibodies were purchased from commercial sources: polyclonal antibodies against PKC α and PKC βII from Santa Cruz Biotechnology; a monoclonal against PKC α from BD Biosciences; phosphoantibodies for Thr^{641} (P641) and Ser^{660} (P660) of PKC βII from Cell Signaling; an anti-HA mAb from Covance; an anti-γ tubulin mAb from Sigma; and polyclonal antibodies against PHLPP1 and PHLPP2 from Bethyl Laboratory. A phospho-specific antibody for Thr^{500} (P500) of PKC βII that specifically recognizes the phosphorylated activation loop of all PKC isozymes was previously generated and characterized (19). Note that the P641 and P660 antibodies are specific for the phosphorylated turn motif and hydrophobic motif, respectively, of PKC and do not recognize constructs with Ala at the phospho-acceptor positions.

Construction of Expression Plasmids—The cloning of human PHLPP1 and PHLPP2 has been described previously (2, 3). There are two splice variants of the PHLPP1 gene, which we named PHLPP1α and PHLPP1β (3). The wild-type PHLPP1 used in this study is PHLPP1α. The expression constructs of HA-tagged full-length wild-type PHLPP1 (HA-PHLPP1), a PH domain deletion mutant of PHLPP1 (HA-ΔPH), a PDZ domain binding mutant of PHLPP1 (HA-ΔC), and full-length wild-type PHLPP2 were generated as described previously (2, 3). The following domains of PHLPP1 were expressed as HA- or GST-tagged fusion proteins in mammalian cells: the PH domain (amino acid residues 1–146), LRR region (amino acid residues 126–652), PP2C domain (amino acid residues 653–906), and the COOH terminus (CT, amino acid residues 907–1205). Relevant regions were amplified using PCR and the PCR products were subcloned into EcoRI and XhoI sites on pcDNA3HA vector (2) or KpnI and NotI sites on pEBG vector (20). Because expression of the HA-tagged PH domain of PHLPP1 was not detected in cells, the PH domain was expressed as a GST fusion protein for the pull-down experiments. The expression plasmids of various GST-tagged domains of PKC βII including N/ψ (NH_2 terminus plus pseudosubstrate), C1A (C1A domain), C1B (C1B domain), C1AB (C1A plus C1B domains), NC1 (NH_2 terminus plus the entire C1 domain), C2 (C2 domain), KD (kinase domain), and CT (COOH terminus) were constructed as described previously (20, 21). The full-length human PP2Cα cDNA was cloned by one-step reverse transcriptase-PCR using human brain total RNA as template. The coding region of PP2Cα was amplified using PCR and subcloned into XhoI and XbaI sites on pcDNA3HA vector.

In Vitro Phosphatase Assay—A GST-tagged fusion protein of PHLPP1-PP2C was expressed and purified as described previously (2). His-tagged PKC βII was expressed and purified from baculovirus-infected Sf21 cells. Briefly, Sf21 cells were maintained in SF-900 II media (Invitrogen) and infected with baculovirus encoding His-PKC βII for 3 days. The infected cells were lysed in phosphate-buffered saline containing 1% Triton X-100 and 10 mM imidazole, and His-PKC βII proteins were purified using nickel-nitrilotriacetic acid beads (Qiagen). The dephosphorylation reactions were carried out in a reaction buffer containing 50 mM Tris (pH 7.4), 1 mM dithiothreitol, and 5 mM $MnCl_2$ at 30 °C for 30 min. The final concentration of His-PKC βII and GST-PP2C in the reactions were 50 and 10 nM, respectively.

Cell Transfection and siRNA Targeting Sequences—COS7, 293T, and H157 cells were maintained in DMEM (Cellgro) con-

taining 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin at 37 °C in 5% CO_2. Transient transfection of all cell types was carried out using Effectene transfection reagents (Qiagen). Lipofectamine 2000 (Invitrogen) was used for transfection of siRNAs. The SmartPool siRNA for each PHLPP isoform is comprised of four individual RNA oligos, and the targeting sequences are as the following: for PHLPP1, 1) GATCTAAGGTTGAACGTAA; 2) TGATCTAGATGCTAT-GATT; 3) GATATTGGCCATAATCAAA; and 4) GAACGC-CTCTGCGAACAAA; for PHLPP2, 1) GGAAAGACCCAGC-TGCATA; 2) GAACTTGTCCCATAATAAA; 3) GCTATAA-TCTTCTCACAGA; and 4) GTACAGCAGTCAACTAATG. The final concentration of the SmartPool siRNAs used in transfections was between 50 and 100 nM. Note that the PHLPP1 siRNAs are targeted against the common regions in both PHLPP1α and PHLPP1β, thus are effective at knocking down all PHLPP1 proteins.

Cell Fractionation—To examine the subcellular localization of PKC, transfected cells were harvested in Buffer A (50 mM Na_2HPO_4, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, 200 μM phenylmethyl-sulfonyl fluoride). The cells were lysed by brief sonication, and the cell lysates were subjected to centrifugation at $16,000 \times g$ for 5 min at 4 °C. The resulting supernatant is referred to as the "detergent-soluble supernatant." The pellet resulting from the centrifugation is referred to as the "detergent-insoluble pellet."

Immunoprecipitation—Immunoprecipitation of PKC βII or HA-PHLPP isoforms was performed as described previously (2). Briefly, the transfected cells were lysed in Buffer A and the detergent-solubilized cell lysate was subjected to immunoprecipitation using the anti-PKC α (which recognizes PKC βII as well) or the anti-HA mAb to precipitate HA-PHLPP1/2. The immunoprecipitates were washed twice in Buffer A and twice in Buffer B (Buffer A plus 250 mM NaCl). Bound proteins were analyzed by SDS-PAGE and immunoblotting.

GST Fusion Protein Pull-down Assay—To examine the interaction between the different domains of PKC βII and PHLPP1, 293T cells were transiently transfected with GST-tagged fusion proteins together with full-length PKC βII or PHLPP1. The transfected cells were lysed in Buffer A, and the detergent-solubilized cell lysates were incubated with glutathione-Sepharose at 4 °C for 2 h. Beads were washed twice in Buffer A and twice in Buffer B, and GST-PH bound beads were analyzed by SDS-PAGE and immunoblotting.

Phorbol Ester-induced Dephosphorylation and Degradation of PKC—To induce dephosphorylation and degradation of endogenous PKC, 293T or H157 cells were treated with 400 nM PDBu or 300 nM PMA for 0–7 h as indicated in figure legends. At each time point, the cells were lysed in SDS sample buffer directly, and the amount of phospho- and total PKC was analyzed by Western blot. Data were analyzed to a first-order decay using the program Kaleidagraph: $y = m1 + m2*\exp(-m3x)$, where y is the fraction of PKC/phospho-PKC and x is time; $m1$ is the offset on the y axis, $m2$ is the fraction of PKC/phospho-PKC that decays, and $m3$ is the rate constant.
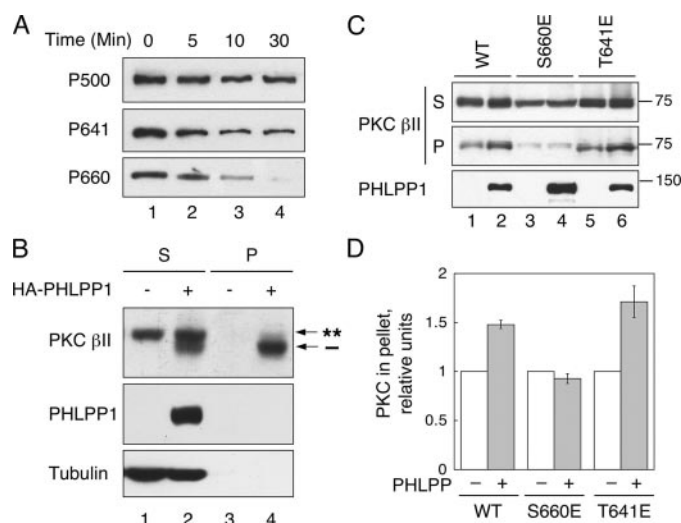

FIGURE 1. **PHLPP1 dephosphorylates PKC *in vitro* and *in vivo*.** *A*, dephosphorylation of PKC βII by purified PP2C domain of PHLPP1. The dephosphorylation reactions were carried out by incubating purified His-PKC βII with purified GST-PP2C for 0–30 min (*lanes 1–4*). The dephosphorylation of PKC βII at Thr^{500}, Thr^{641}, or Ser^{660} was detected by the phospho-specific antibodies P500, P641, or P660, respectively. *B*, dephosphorylation of PKC βII upon overexpression of HA-PHLPP1 in cells. COS7 cells were transfected with PKC βII together with either vector (*lanes 1* and *3*) or HA-PHLPP1 (*lanes 2* and *4*). The cells were fractionated into detergent-soluble supernatants (S, *lanes 1* and *2*) and detergent-insoluble pellets (P, *lanes 3* and *4*). The appearance of faster migrating species of PKC βII indicates dephosphorylation of the protein (labeled by a *dash*), the mature fully phosphorylated species is labeled with *two asterisks*. The expression of PKC βII and HA-PHLPP1 was detected by the anti-PKC βII antibody (*upper panel*) and the anti-HA mAb (*middle panel*), respectively. Tubulin was detected with an anti-γ tubulin mAb (*lower panels*). *C*, accumulation of WT and mutant PKC βII in pellet fractions upon overexpression of HA-PHLPP1. COS7 cells expressing PKC βII WT (*lanes 1* and *2*), S660E (*lanes 3* and *4*), or T641E (*lanes 5* and *6*) together with either vector (*lanes 1*, *3*, and *5*) or HA-PHLPP1 (*lanes 2*, *4*, and *6*) were fractionated into detergent-soluble supernatants (S, *upper panel*) and detergent-insoluble pellets (*P*, *middle panel*). The expression of WT and mutant PKC βII was detected using the anti-PKC βII antibody. The presence of HA-PHLPP1 was detected using the anti-HA mAb (*lower panel*). *D*, graph showing quantified results of accumulation of detergent-insoluble PKC βII in pellet fractions upon PHLPP1 overexpression. Western blots as shown in *C* were scanned and quantified using a Bio-Rad GS-800 densitometer. For cells co-expressing WT or mutant PKC with the vector (control cells), the amount of PKC in the detergent-insoluble pellet fractions was set to 1. The relative amount of PKC in cells co-expressing HA-PHLPP1 was normalized to that of the control cells. Data represent the mean ± S.E. of three independent experiments.

RESULTS

PHLPP1 Dephosphorylates PKC βII in Vitro and in Vivo— We have previously shown that the isolated PP2C domain of PHLPP1 efficiently dephosphorylates Akt *in vitro* (2). Here, we first addressed whether the isolated PP2C domain of PHLPP1, freed of any inhibitory constraints, had catalytic activity toward pure, phosphorylated PKC βII *in vitro*. As described previously, the PP2C domain of PHLPP1 was expressed as a GST-tagged fusion protein and purified from bacteria (2). Full-length PKC βII was purified from recombinant baculovirus-infected insect cells; this PKC is fully phosphorylated at this PKC is fully phosphorylated at the two conserved C-terminal sites, Thr^{641} and Ser^{660}, and partially phosphorylated at the activation loop site, Thr^{500} (17). The Western blot in Fig. 1*A* shows that the PP2C domain effectively dephosphorylated the hydrophobic motif site, Ser^{660}, and the turn motif, Thr^{641}. In contrast, the activation loop site Thr^{500} was relatively resistant to dephosphorylation by the PP2C domain of PHLPP1 *in vitro*. These results
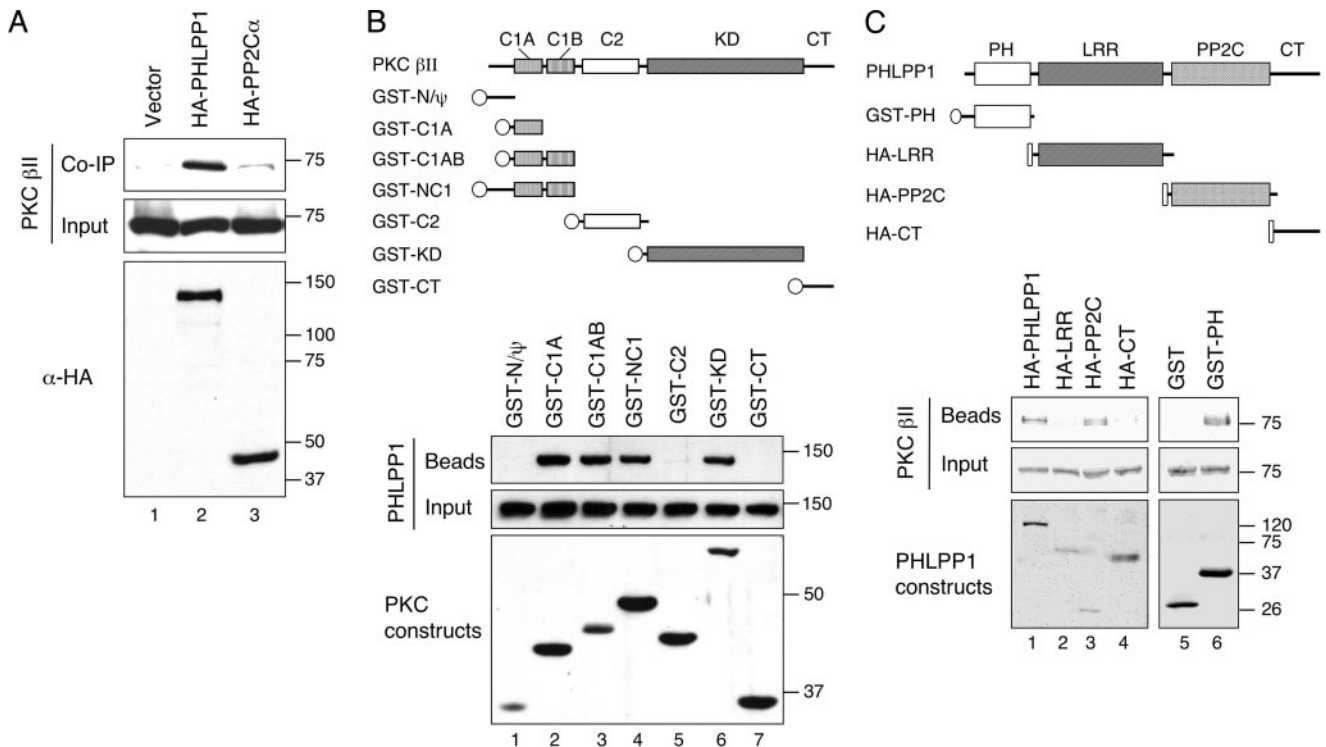
FIGURE 2. **PHLPP1 interacts with its substrate PKC.** *A*, co-immunoprecipitation (*Co-IP*) of PHLPP1 and PKC βII. COS7 cells were co-transfected with PKC βII and vector, HA-PHLPP1, or HA-PP2Cα (*lanes 1–3*, respectively). The detergent-soluble supernatants were immunoprecipitated with the anti-HA mAb, and immunoprecipitated proteins were analyzed using SDS-PAGE and immunoblotting. The presence of PKC βII in the immune complexes (*co-IP*, *upper panel*) and in 10% of lysates used in the immunoprecipitation (*input*, *middle panel*) was detected using the anti-PKC βII antibody, and the amount of HA-tagged proteins in the immunoprecipitates was detected by the anti-HA high affinity rat mAb (*lower panel*). *B*, PHLPP1 binds to the C1A and the kinase domains of PKC βII. The GST-tagged different domains of PKC βII including N/ψ, C1A, C1AB, NC1, C2, kinase dead (KD), and CT (*lanes 1–7*, respectively) were co-expressed with HA-tagged PHLPP1 in 293T cells. The GST-tagged fusion proteins were pulled down using glutathione-Sepharose, and the precipitated proteins were analyzed using SDS-PAGE and immunoblotting. The presence of HA-PHLPP1 bound to the beads (*beads*, *upper panel*) and in 10% of lysates (*input*, *middle panel*) was detected using the anti-HA mAb. Different GST fusion proteins bound to the beads were detected using the anti-GST antibody (*lower panel*). *C*, PKC βII binds to the PH and the PP2C domains of PHLPP1. PKC βII was co-expressed with HA-PHLPP1, HA-LRR, HA-PP2C, HA-CT, GST expression vector, or GST-PH (*lanes 1–6*, respectively) in 293T cells. The detergent-solubilized lysates were immunoprecipitated with the anti-HA mAb (*lanes 1–4*) or incubated with glutathione-Sepharose beads (*lanes 5 and 6*). The proteins bound to the beads were separated on a SDS-PAGE gel and analyzed using immunoblotting. The presence of PKC βII bound to the beads (*beads*, *upper panel*) and in 10% of lysates (*input*, *middle panel*) was detected using the anti-PKC βII antibody. Different domains of PHLPP1 precipitated were detected using the anti-HA mAb (*lanes 1–4*) or the anti-GST antibody (*lanes 5 and 6*).

reveal that the PP2C domain of PHLPP1 dephosphorylates the hydrophobic motif site and the turn motif of PKC *in vitro*.

To ask whether PHLPP1 regulates PKC *in vivo*, we co-expressed HA-PHLPP1 or vector plasmid with PKC βII in 293T cells and examined the subcellular distribution of PKC βII. The Western blots in Fig. 1*B* show the partitioning of PKC βII in the detergent-soluble supernatant (S) and detergent-insoluble pellet (P) fractions of cells. As reported previously, the majority of the PKC βII in control cells partitioned in the detergent-soluble supernatant and migrated as an upper band (*double asterisk*) corresponding to the mature species that is quantitatively phosphorylated at the two COOH-terminal sites (phosphorylation of these sites causes mobility shifts) and mostly phosphorylated at the PDK-1 site (22). In contrast, co-expression of PHLPP1 resulted in the appearance of a faster-migrating species of PKC βII (*dash*) corresponding to dephosphorylated PKC in the detergent-soluble fraction (Fig. 1*B*, *lane 2*). This dephosphorylated species accumulated in the detergent-insoluble pellet (*lane 4*). Note that the dephosphorylation (and phosphorylation) of the turn motif and hydrophobic motif are tightly coupled. Thus, species of intermediate mobility, reflecting loss of phosphate at only one COOH-terminal site, are not detectable. These results reveal that co-expression of PHLPP1 with PKC βII in cells pro-

motes the dephosphorylation and accumulation of dephosphorylated PKC in the detergent-insoluble fraction of cells.

To dissect the role of negative charge at the turn motif (Thr[641]) or the hydrophobic motif (Ser[660]) in the PHLPP-mediated distribution of PKC to the detergent-insoluble pellet, we examined two phosphomimetics of PKC βII: T641E and S660E. Fig. 1*C* shows that the PHLPP-mediated redistribution was not affected by negative charge on Thr[641] but was prevented when negative charge was locked on Ser[660] (quantified data shown in Fig. 1*D*). These data suggest that loss of negative charge on Ser[660], but not Thr[641], shunts PKC to the detergent-insoluble fraction.

PHLPP1 Interacts with Its Substrate PKC βII—Protein phosphatases are known to form complexes with their substrates as mechanisms to ensure specificity (23). To explore whether PHLPP1 interacts with PKC βII in cells, we performed co-immunoprecipitation experiments using cells co-expressing PKC βII and HA-PHLPP1. As negative controls, PKC βII was co-expressed with either a vector plasmid or an expression construct of HA-tagged human PP2Cα (HA-PP2Cα). Fig. 2*A* shows that PKC βII was readily detected in the immunoprecipitates of HA-PHLPP1 (*lane 2*). However, only background levels of PKC βII were present in the immunoprecipitates of vector

alone (*lane 1*) or HA-PP2Cα (*lane 3*). Note that the "input" blot in Fig. 2*A* shows the phosphorylated species of PKC in the detergent-soluble fraction; analysis of the detergent-insoluble pellet revealed robust accumulation of dephosphorylated PKC in cells expressing HA-PHLPP1 but not vector or HA-PP2Cα (data not shown). Importantly, these co-immunoprecipitation studies reveal that PKC βII specifically associates with PHLPP1, and not another member of the PP2C family of phosphatases, PP2Cα, in cells.

To delineate the regions in PKC βII that mediate the binding to PHLPP1, GST pull-down experiments were performed using cells expressing various GST-tagged domains of PKC βII and HA-PHLPP1. The data in Fig. 2*B* show that PHLPP1 bound to all constructs containing the C1A domain (GST-C1A, *lane 2*; GST-C1AB, *lane 3*; GST-NC1, *lane 4*), as well as the kinase domain core (GST-KD, *lane 6*). It did not bind a construct of the extreme amino terminus, including the pseudosubstrate sequence (*lane 1*), the C2 domain (*lane 5*), or the extreme COOH terminus (*lane 7*). Thus, the C1A domain and kinase core provide the major determinants that mediate the binding of PKC to PHLPP1.

We next tested which domain(s) in PHLPP1 mediate(s) the binding to PKC βII. The leucine-rich repeat (LRR) region, the PP2C domain, and the COOH terminus of PHLPP1 were expressed as HA-tagged fusion proteins, and the PH domain of PHLPP1 was expressed as a GST-tagged fusion protein. Cells expressing PKC βII together with HA-PHLPP1, HA-LRR, HA-PP2C, HA-CT, or GST-PH were subjected to immunoprecipitation or GST pull-down (Fig. 2*C*). PKC βII co-immunoprecipitated with HA-PP2C and GST-PH of PHLPP1 (*lanes 3* and *6*) as well as the full-length HA-PHLPP1 (*lane 1*). Although the HA-PP2C domain expressed at considerably lower levels than full-length HA-PHLPP1 or the PH domain, the amount of PKC associated with all three constructs was comparable. This suggests that the isolated PP2C domain contains the primary determinants driving the interaction with PKC and that this interaction is weakened in the context of the full-length protein. In addition, weak binding to the PH domain stabilizes the interaction of PHLPP1 with PKC.

The PH Domain of PHLPP1 Is Required for Dephosphorylation of PKC βII—To explore the role of potential regulatory regions in PHLPP1, we examined the effect of deleting either the PH domain or PDZ-binding motif on the ability of PHLPP1 to promote the accumulation of dephosphorylated PKC in the detergent-insoluble fraction of cells. As described in Figs. 1 and 3, co-expression of WT PHLPP1 with PKC βII resulted in the accumulation of a faster-migrating species of PKC βII (Fig. 3*A*, *dash*) in the detergent-insoluble fraction of the cells (Fig. 3*A*, *lane 6*) that was absent in vector co-transfected cells (Fig. 3*A*, *lane 5*). A similar, but significantly enhanced, accumulation of PKC in the detergent-insoluble fraction was observed in cells transfected with a construct of PHLPP1 deleted in the COOH-terminal PDZ-binding motif (ΔC, *lanes 4* and *8*). In contrast, deletion of the PH domain suppressed the ability of PHLPP1 to dephosphorylate PKC βII and relocalize the dephosphorylated species to the detergent-insoluble fraction (ΔPH, *lane 7*). Thus, the PH domain is nec-
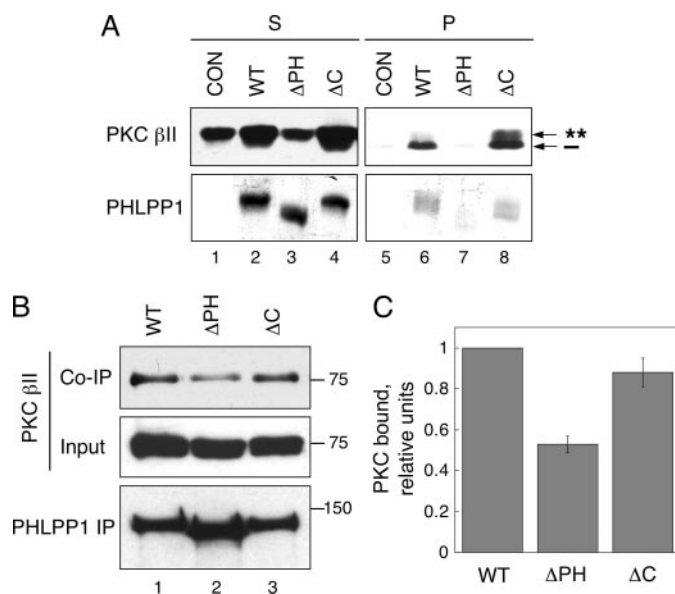

FIGURE 3. **The PH domain of PHLPP1 plays a critical role in PHLPP-mediated dephosphorylation of PKC.** *A*, the PH domain of PHLPP1 is required for efficient dephosphorylation of PKC βII *in vivo*. COS7 cells were transfected with PKC βII together with vector (si-CON, *lanes 1* and *5*), HA-PHLPP1 (WT, *lanes 2* and *6*), HA-ΔPH (ΔPH, *lanes 3* and *7*), or HA-ΔC (ΔC, *lanes 4* and *8*). The transfected cells were fractionated into detergent-soluble supernatants (*S*, *lanes 1–4*) and detergent-insoluble pellets (*P*, *lanes 5–8*), and analyzed using SDS-PAGE and immunoblotting. The expression of PKC βII and different PHLPP1 constructs was detected using the anti-PKC βII antibody and the anti-HA mAb, respectively. *B*, deletion of the PH domain in PHLPP1 results in reduced association between PHLPP1 and PKC βII. COS7 cells expressing PKC βII together with WT HA-PHLPP1, HA-ΔPH, or HA-ΔC (*lanes 1–3*, respectively) were lysed and subjected to immunoprecipitation using the anti-HA mAb. The amount of PKC βII co-immunoprecipitated with different PHLPP1 constructs is shown in the *upper panel*, and 10% of lysates used in the immunoprecipitation is shown in the *middle panel* as input (detected by the anti-PKC βII antibody). The presence of WT and mutant PHLPP1 constructs in the immunoprecipitates was detected using the anti-HA high affinity rat mAb (*lower panel*). *C*, graph showing quantified results of relative binding between PKC βII and different PHLPP1 constructs. Western blots from experiments described in *B* were scanned and quantified using a Bio-Rad GS-800 densitometer. The amount of PKC βII bound to WT PHLPP1 was set to 1, and the relative amount of PKC βII bound to PHLPP1 mutants was normalized to that of WT. Data represent the mean ± S.E. of three independent experiments.

essary for efficient dephosphorylation of PKC by PHLPP in cells.

We next investigated whether deletion of the PH domain or the PDZ-binding motif in PHLPP1 affected its interaction with PKC βII. Co-immunoprecipitation experiments demonstrated that deletion of the PH domain resulted in a 50% reduction of the amount of PKC βII bound to PHLPP1 (Fig. 3, *B*, *lane 2*, and *C*). This finding is consistent with the PH domain of PHLPP1 providing a docking site for binding PKC βII as shown in Fig. 2*C*. In contrast, deletion of the PDZ-binding motif had no significant effect on the interaction of PHLPP1 with PKC βII (Fig. 3, *B*, *lane 3*, and *C*). These data reveal that the PH domain, and not the PDZ-binding motif, controls the interaction of PHLPP1 with PKC.

The Second Isoform of the PHLPP Family, PHLPP2, Dephosphorylates PKC βII—Because there are two functionally related isoforms in the PHLPP family, we next addressed whether the second isoform, PHLPP2, also dephosphorylates PKC. Fig. 4*A* shows that co-expression of HA-PHLPP2 increased the amount of dephosphorylated PKC βII in the detergent-insolu-
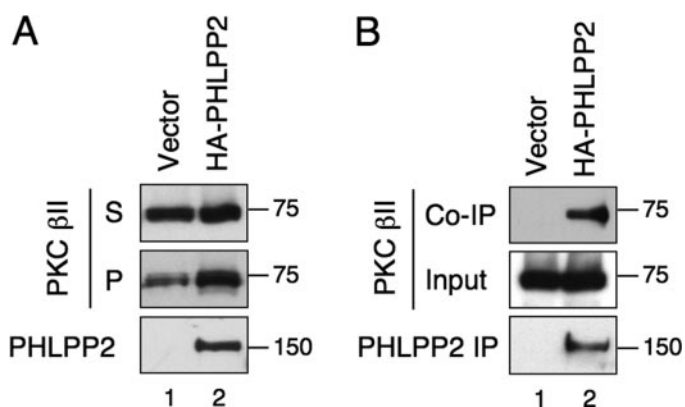
A

B

FIGURE 4. **The second isoform of the PHLPP family, PHLPP2, functions as a phosphatase for PKC.** *A*, dephosphorylation of PKC βII upon overexpression of HA-PHLPP2 in cells. COS7 cells were transfected with PKC βII together with either vector (*lane 1*) or HA-PHLPP2 (*lane 2*). The cells were fractionated into detergent-soluble supernatants (*S, upper panel*) and detergent-insoluble pellets (*P, middle panel*). The expression of PKC βII and HA-PHLPP2 was detected by the anti-PKC βII antibody (*upper panel*) and the anti-HA mAb (*middle panel*), respectively. *B*, co-immunoprecipitation (*Co-IP*) of PKC βII and HA-PHLPP2. COS7 cells were co-transfected with PKC βII and vector or HA-PHLPP2 (*lanes 1* and *2*, respectively). The detergent-solubilized lysates were immunoprecipitated with the anti-HA mAb, and immunoprecipitated proteins were analyzed using SDS-PAGE and immunoblotting. The presence of PKC βII in the immune complex (*co-IP, upper panel*) and in 10% of lysates used in the immunoprecipitation (*input, middle panel*) was detected using the anti-PKC βII antibody, and the amount of HA-PHLPP2 in the immunoprecipitates was detected by the anti-HA high affinity rat mAb (*lower panel*).

ble fraction of the cells (*lane 2*). Furthermore, co-immunoprecipitation experiments showed that PKC βII specifically interacted with PHLPP2 in cells (Fig. 4*B*, *lane 2*). Taken together, these results suggest that PHLPP2 functions similarly to PHLPP1 in dephosphorylating PKC βII *in vivo*.

Phorbol Ester-induced Dephosphorylation of the Hydrophobic Motif Site of PKC Is Okadaic Acid-insensitive—To begin to assess the role of PHLPP in the phorbol ester-induced dephosphorylation of PKC, we first examined how the phosphorylation sites in the COOH terminus of PKC become dephosphorylated upon PDBu treatment. Because PKC α is the major conventional PKC in 293T cells, we examined the effect of PHLPP knockdown on this isoform; similar to PKC βII, PKC α is efficiently dephosphorylated by overexpressed PHLPP in cells (data not shown). 293T cells were treated with PDBu for 1 h or left untreated, and the cell lysates were analyzed for the dephosphorylation of endogenous PKC α. Fig. 5*A* shows that the intensity of the labeling by the phospho-specific antibodies for both the hydrophobic motif and turn motif sites decreased upon PDBu treatment (*upper* and *middle panels*) revealing loss of phosphate at both sites; PKC α is the major P660 and P641-reacting species in 293T cells, however, novel isoforms also contribute to this signal. In addition, the appearance of faster migrating bands detected with the anti-PKC α antibody supported the dephosphorylation of PKC α (Fig. 5*A*, *bottom panel*). (Note that although phorbol esters have been reported to slow the maturation of PKC (15, 24), pulse-chase analysis revealed that the faster migrating band detected in our experiments (*lane 2*) is mature PKC that has been dephosphorylated (data not shown).) Under the conditions of our experiments, PDBu-induced dephosphorylation of the activation loop site (Thr^{497} in PKC α) was not observed (data not shown, see Fig. 5, *B* and *C*,

as well). Note that an intermediate species (*single asterisk*) was detected by the phospho-specific antibody for phospho-Thr^{641}, but not phospho-Ser^{660}, indicating that dephosphorylation of Ser^{660} precedes that of Thr^{641}. This is consistent with mass spectrometric analysis revealing that a minor species of PKC phosphorylated at Thr^{641} but not Ser^{660} can be detected in cells (17). These data suggest that the phosphatase responsible for dephosphorylating the hydrophobic motif site of PKC promotes the termination phase of PKC signaling. The data presented so far suggest that PHLPP is the phosphatase controlling the phosphorylation state of hydrophobic motif site of PKC.

PP2C family members are not inhibited by okadaic acid (OA) (25), leading us to test whether PDBu-induced dephosphorylation of PKC is sensitive to OA treatment. We analyzed the phosphorylation state at each of the three conserved phosphorylation sites on PKC βII in cells treated with or without phorbol esters and with or without OA. Previous studies have shown that phosphorylation at the activation loop site (Thr^{500}) is required for processing of PKC; however, phosphorylation at this site becomes dispensable once PKC is fully matured and only half of the pool of mature, signaling-competent PKC is phosphorylated at this position basally in cells (17). Consistent with this, Fig. 5*B* and the corresponding graph quantifying the data from three separate experiments (Fig. 5*C*) show that treating cells with OA resulted in an ~2-fold increase in the phosphorylation at Thr^{500} for both WT and the S660E mutant of PKC βII compared with untreated control cells (*P500 panels*, *lanes 1, 2, 5,* and *6*). Similarly, Thr^{500} was phosphorylated to a 2-fold higher level in cells treated with OA prior to PDBu treatment; PDBu treatment alone did not induce significant dephosphorylation at Thr^{500} under the conditions of this experiment (*lanes 3* and *7*). These results are consistent with the dephosphorylation of the activation loop Thr^{500} site of PKC being catalyzed by an OA-sensitive phosphatase. Because the majority of WT PKC βII is fully phosphorylated at both COOH-terminal sites basally (17), the phosphorylation of both the Thr^{641} and Ser^{660} sites in WT PKC βII was not affected by OA treatment alone (Fig. 5*B*, *P641* and *P660 panels*, *lanes 1* and *2*; Fig. 5*C*, *columns 5, 6* and *9, 10*). To examine the effect of OA on these sites, we took two approaches. First, we took advantage of the S600E mutant of PKC: the mutant with Glu (rather than phosphate) at this position has increased sensitivity to dephosphorylation resulting in the presence of some protein that is not phosphorylated at the Thr^{641} site in cells (10, 26). Thus, because it is not completely phosphorylated at the turn motif, the S660E mutant can be used to test the OA sensitivity of the Thr^{641} site. Fig. 5*C* shows that the phosphorylation state of Thr^{641} in the S660E mutant increased 1.3-fold upon OA treatment compared with untreated cells (*columns 17* and *18*). As a second approach, we examined the effect of OA on PDBu-induced dephosphorylation of the turn and hydrophobic motifs. Under conditions of the experiment, PDBu caused ~55% of the turn motif and 65% of the hydrophobic motif to become dephosphorylated (*columns 7* and *11*). Importantly, OA inhibited the dephosphorylation of the turn motif by ~50% (compare *columns 7* and *8*) but had no effect on the dephosphorylation of the hydrophobic motif (compare *columns 11* and *12*). The PDBu-induced dephosphorylation of Thr^{641} in the S660E
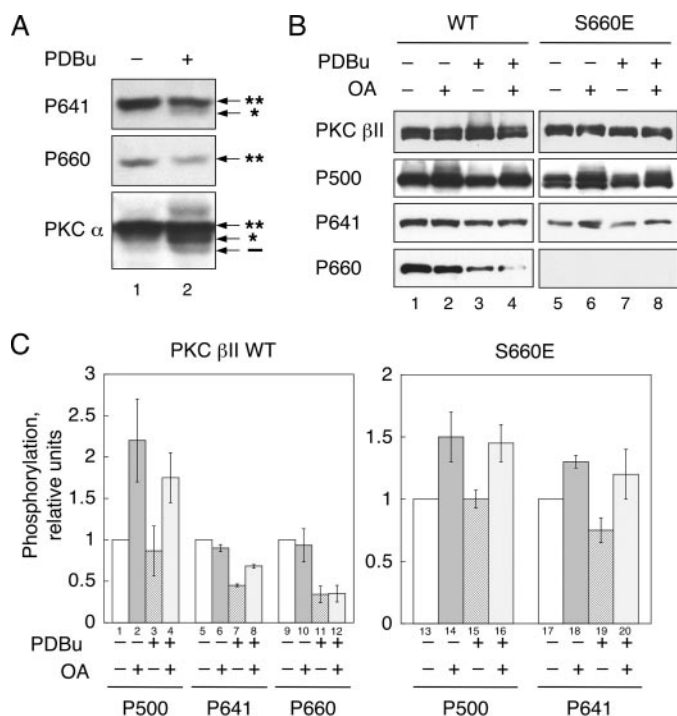
FIGURE 5. **Dephosphorylation of the hydrophobic motif site of PKC upon phorbol ester treatment is OA-insensitive.** *A*, the hydrophobic motif site of PKC is the first site dephosphorylated upon PDBu treatment. 293T cells were treated with PDBu (400 nM) for 0 and 1 h (*lanes 1* and *2*). The detergent-soluble supernatants were analyzed for dephosphorylation of endogenous PKC α using immunoblotting. The phosphorylation state of PKC α was probed using the phospho-specific P641 and P660 antibodies, and total protein was detected using the anti-PKC α antibody. The dephosphorylated PKC α is labeled with a *dash*, the intermediate species (phosphate on one of the two COOH-terminal phosphorylation sites) is labeled with *one asterisk*, and the fully phosphorylated species (phosphates on both COOH-terminal phosphorylation sites) is labeled with *two asterisks*. *B*, PDBu-induced dephosphorylation of the hydrophobic motif site of PKC βII is OA-insensitive. COS7 cells expressing PKC βII WT (*lanes 1– 4*) or S660E mutant (*lanes 5– 8*) were subjected to one of the following treatments: Me$_2$SO (*lanes 1* and *5*), OA (200 nM) for 30 min (*lanes 2* and *6*), PDBu (400 nM) for 2 h (*lanes 3* and *7*), or pretreatment with OA (200 nM) for 30 min then PDBu (400 nM) for an additional 2 h (*lanes 4* and *8*). Detergent-soluble supernatants were prepared from the treated cells and analyzed using SDS-PAGE and immunoblotting. The phosphorylation state of PKC βII was probed using the phospho-specific P500, P641, and P660 antibodies, and the total protein was detected using the anti-PKC βII antibody. *C*, graphs showing quantified results of PKC βII WT and S660E phosphorylation in response to OA and PDBu treatment. Western blots from experiments as in *B* were scanned and quantified using a Bio-Rad GS-800 densitometer. The ECL signals generated by the P500, P641, and P660 antibodies were normalized to the amount of total PKC proteins as detected by the anti-PKC βII antibody. The basal phosphorylation at all three sites was set to 1, and the relative phosphorylation upon different treatments was obtained by normalizing to the basal phosphorylation. Data represent the mean $\pm$ S.E. of three independent experiments.

mutant was similarly inhibited by OA pretreatment (Fig. 5*C*, compare *columns 19* and *20*). These data establish that the dephosphorylation of Thr[641], but not Ser[660], is controlled by an OA-sensitive phosphatase. This insensitivity of the phosphorylation state of Ser[660] to OA is consistent with PHLPP being the key protein phosphatase for the hydrophobic motif.

Depletion of Endogenous PHLPP Isoforms Inhibits Phorbol Ester-induced Dephosphorylation of PKC α—To elucidate the role of endogenous PHLPP isoforms in regulating phorbol ester-induced dephosphorylation of PKC in cells, siRNAs specifically targeting PHLPP1 or PHLPP2 were used to deplete cells of PHLPP. We used H157 cells for the siRNA experiments

because we have previously shown that PHLPP1 can be effectively knocked down in this cell line (2). Similar to experiments with 293T cells, we monitored the dephosphorylation process of endogenous PKC α in H157 cells because it is the predominant PKC isoform expressed. The *top two panels* in Fig. 6*A* show that the expression of both endogenous PHLPP1 and PHLPP2 was effectively reduced in H157 cells transfected with specific siRNAs (*lanes 3– 8*). Treating cells with PDBu for 2 h did not cause dephosphorylation of the activation loop site of endogenous PKC α in the control- or PHLPP-specific siRNA-transfected cells (*P500 panel*). Under the same conditions, ~40–50% of the turn motif site became dephosphorylated (*P641 panel*). However, the extent of dephosphorylation of PKC α at this site was not significantly different in the control- or PHLPP-specific siRNA-transfected cells (Fig. 6*B*). In contrast, the PDBu treatment resulted in an ~50% reduction of hydrophobic motif site phosphorylation of endogenous PKC α in the control siRNA-transfected cells (*P660 panel*, *lanes 1* and *2*), and knocking down either PHLPP1 or PHLPP2 or both prevented this dephosphorylation (Fig. 6*A*, *lanes 3– 8*, and *B*). Interestingly, depletion of either isoform of PHLPP was sufficient to inhibit dephosphorylation of the hydrophobic motif site and to maintain PKC α in a fully phosphorylated state. Thus, no additive effect was observed upon depletion of both PHLPP isoforms. In addition, no significant loss of total PKC α protein was observed in the time frame of these experiments, consistent with the notion that dephosphorylation precedes protein degradation of PKC (*PKC α panel*). As a negative control, the amount of endogenous PKC ζ, a phorbol ester-resistant PKC isoform, was not significantly affected by PDBu treatment (*PKC ζ panel*). These data reveal that both endogenous PHLPP1 and PHLPP2 catalyze the PDBu-induced dephosphorylation of PKC α *in vivo* and this PHLPP-mediated dephosphorylation is specific for the hydrophobic motif site of PKC.

To further examine the effect of PHLPP-mediated dephosphorylation in modulating the degradation process of PKC after prolonged activation, the time course of endogenous PKC α down-regulation was examined in PHLPP knockdown cells. PMA was used here to treat cells because it is more potent than PDBu in inducing degradation of endogenous PKC. 293T cells transfected with control siRNA or siRNAs targeting both PHLPP1 and PHLPP2 isoforms were treated with PMA for 0 –7 h. Fig. 6*C* shows that phorbol ester treatment resulted in the dephosphorylation of PKC and an accompanying reduction in the total amount of PKC. Fitting the data from three independent experiments to a first-order decay revealed that the half-time for the dephosphorylation at both Thr[641] and Ser[660] as well as the degradation of PKC was the same and ~1 h. Genetic depletion of PHLPP1 and PHLPP2 had no significant effect on the loss of phosphate at Thr[641]: the half-time for dephosphorylation of this site was 0.9 $\pm$ 0.3 h for the PHLPP knockdown compared with 0.7 $\pm$ 0.5 h for control) (Fig. 6, C, *P641 panel*; *D*, *P641 graph*). However, the dephosphorylation of Ser[660] was markedly reduced in cells depleted of PHLPP compared with control cells. Most noticeably, fitting the data to an exponential decay revealed that half as much PKC was dephosphorylated on Ser[660] in cells lacking PHLPP compared with control cells: 44 $\pm$ 4% of the PKC was dephosphorylated in cells depleted of
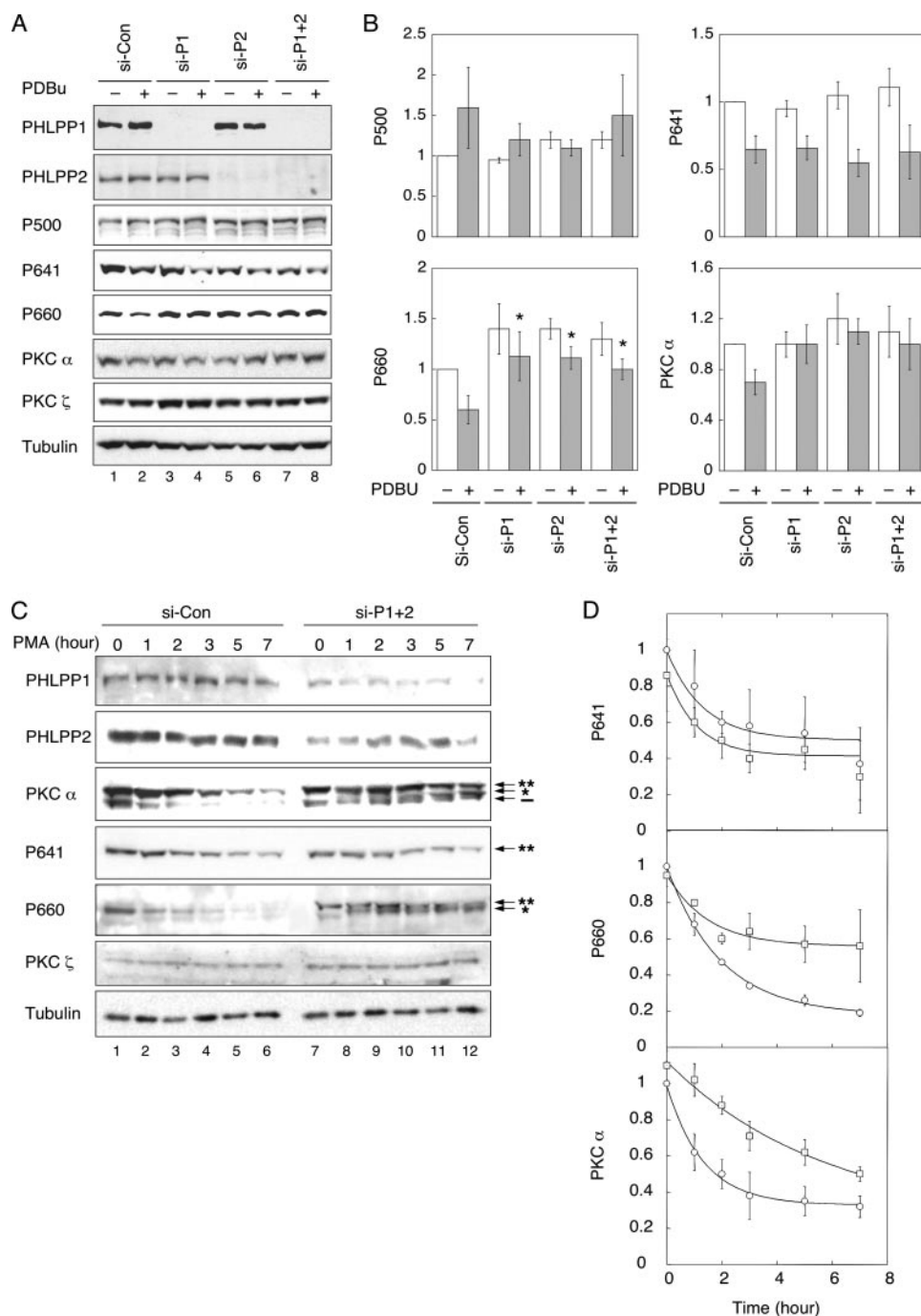
FIGURE 6. **Depletion of endogenous PHLPP isoforms attenuates phorbol ester-induced dephosphorylation of PKC.** *A*, dephosphorylation of endogenous PKC α upon PDBu treatment in cells expressing control or PHLPP isoform-specific siRNAs. H157 cells were transfected with control siRNA (*si-Con*, *lanes 1* and *2*) or siRNAs against PHLPP1 (*si-P1*, *lanes 3* and *4*), PHLPP2 (*si-P2*, *lanes 5* and *6*), or both PHLPP isoforms (*si-P1 + 2*, *lanes 7* and *8*). The cells were treated with Me$_2$SO (*lanes 1*, *3*, *5*, and *7*) or 400 nM PDBu (*lanes 2*, *4*, *6*, and *8*) for 2 h. The detergent-solubilized lysates were prepared and analyzed using SDS-PAGE and immunoblotting. The expression of endogenous PHLPP1 and PHLPP2 was detected using the anti-PHLPP1 and anti-PHLPP2 antibodies. The phosphorylation state of endogenous PKC α was probed with the phospho-specific P500, P641, and P660 antibodies. The total PKC α and PKC ζ proteins were detected using the anti-PKC α and anti-PKC ζ antibodies, respectively. Tubulin was detected using the anti-γ tubulin mAb. *B*, graphs showing relative levels of total PKC and phosphorylated PKC following PDBu treatment of cells treated with control siRNA or PHLPP-specific siRNA from four independent experiments. Western blots as shown in *A* were scanned and quantified using a Bio-Rad GS-800 densitometer. Data are normalized to tubulin levels. The relative phosphorylation for control cells under the basal condition (without PDBu treatment) was set to 1, and all other conditions were compared accordingly. Data represent the mean ± S.E. The *asterisks* in the P660 graph indicate $p < 0.05$. *C*, the time course of PMA-induced degradation of endogenous PKC α in 293T cells expressing control (*lanes 1–6*) or PHLPP specific (*lanes 7–12*) siRNAs. Whole cell lysates were prepared from cells transfected with control or PHLPP-specific siRNA (a combination of siRNAs for both PHLPP isoforms) and analyzed by Western blot. The expression of endogenous PHLPP1, PHLPP2, PKC α, PKC ζ, and tubulin was detected as described in *A*. The phosphorylation status of PKC on the turn and hydrophobic motifs was detected using the P641 and P660 antibodies, respectively. *Double asterisk* indicates the position of PKC phosphorylated at both COOH-terminal sites, *single asterisk* denotes PKC phosphorylated at only one COOH-terminal site, and *dash* represents PKC that is not phosphorylated at either COOH-terminal site. *D*, summarized results of three independent experiments as described in *C*. The ECL signals of P641, P660, anti-PKC α, and anti-tubulin antibodies were quantified using a FluorChem digital imager (Alpha Innotech), and the amount of phospho- and total-PKC α at each time point was obtained by normalizing the amount of P641, P660, or PKC α to tubulin. Data were fit to a monoexponential curve to obtain the half-time and fraction of PKC dephosphorylated or degraded. Data represent the mean ± S.E. ($n = 3$).

PHLPP compared with 82 ± 2% dephosphorylated in control cells (Fig. 6D; note where curves plateau). The accumulation of an intermediate-migrating band (Fig. 6C, labeled with *single asterisk*) in PHLPP knockdown cells represented the large fraction of PKC phosphorylated at only Ser^{660} and not Thr^{641} (this intermediate band was not labeled by the P641 antibody). Consistent with PHLPP knockdown rendering a pool of PKC resistant to degradation, the half-life of PKC α degradation was slowed to 4 ± 2 h in the knockdown cells compared with 0.91 ± 0.08 in control cells. Note that the P660 antibody recognizes all PKC isoforms so that the difference in fraction of PKC α resistant to degradation (33 ± .02%) *versus* fraction of PKC resistant to dephosphorylation on Ser^{660} (56 ± 4%) likely accounts for slight variations in the sensitivity to PHLPP dephosphorylation of the various isoforms. Thus, a significant fraction of PKC was resistant to dephosphorylation at Ser^{660} but not Thr^{641} in cells depleted of PHLPP isoforms. The expression level of a phorbol ester-insensitive PKC isoform, PKC ζ, was not altered by PMA treatment in either control or PHLPP knockdown cells. Taken together, these results demonstrate that both PHLPP isoforms mediate the dephosphorylation of endogenous PKC on the hydrophobic motif following activation-induced down-regulation. They also show that this dephosphorylation is required for one pathway of phorbol ester-mediated down-regulation of PKC.

Expression of Endogenous PKC Isoforms Is Modulated by PHLPP Expression—PKC levels are elevated in a number of cancers, notably colon (27, 28). To directly address whether PHLPP levels control PKC levels in colon cancer cell lines, we specifically depleted PHLPP1, PHLPP2, or both PHLPP isoforms from a colon cancer cell line that has relatively high PHLPP expression, the DLD-1 cell line. Fig. 7A reveals that depletion of either isoform alone, or both, caused a 3-fold increase in the levels of PKC βII. PKC α levels were not significantly affected by PHLPP knockdown, nor were PKC ζ levels altered. Single knockdown caused a very modest increase in PKC δ levels, an increase that was more pronounced when both PHLPP1 and PHLPP2 were depleted. These data reveal PHLPP selectively controls the levels of PKC βII in the DLD-1 colon cancer cell line.

To further determine whether the basal expression of endogenous PKC in normal cells is regulated by PHLPP, we transfected two normal breast epithelial cell lines with control or PHLPP-specific siRNAs and monitored the expression level of PKC. The expression of both PHLPP isoforms was effectively knocked down by the combination of PHLPP-specific siRNAs in those cells (Fig. 7B, *PHLPP1* and *PHLPP2 panels*). Importantly, knockdown of PHLPP1 and PHLPP2 caused a marked increase in the levels of PKC α, PKC βII, and the novel isoform PKC ε compared with the control cells (Fig. 7A, *PKC α*, *PKC βII*, and *PKC ε panels*). These results suggest that PHLPP is involved in the constitutive turnover process of conventional and novel PKC isoforms in normal cells, consistent with the finding that PHLPP-mediated dephosphorylation controls the degradation process of PKC.

DISCUSSION

The phosphorylation state of PKC controls its stability, as best illustrated by the finding that the enzyme is depleted in cells lacking the upstream kinase, PDK-1 (29). Because the phosphorylation of PKC is constitutive, dephosphorylation mechanisms are poised to play a key role in controlling the amount of PKC and thus the amplitude of the PKC signal. Here, we identified the novel protein phosphatases PHLPP1 and PHLPP2 as playing major roles in regulating the dephosphorylation of PKC in cells. We showed that both phosphatases bind and dephosphorylate PKC βII on the hydrophobic motif, an event that shunts PKC βII to the detergent-insoluble fraction of cells (Fig. 8). In addition, depletion of endogenous PHLPP1 or PHLPP2 via siRNA inhibits phorbol ester-induced dephosphorylation of the hydrophobic motif site on PKC α, revealing that both PHLPP isoforms are endogenous negative regulators of PKC signaling. Underscoring a key role of PHLPP in normal physiology, knockdown of PHLPP isoforms in a colon cancer cell line with relatively high PHLPP expression causes a 3-fold increase in the expression of PKC βII, an isoform whose levels are elevated in colon cancer (27). In addition, knockdown of PHLPP in normal breast epithelial cells results in a marked increase of endogenous PKC expression. Thus, PHLPP isozymes control the level of cellular PKC by dephosphorylating a key site, the hydrophobic motif, that controls the stability of this family of kinases.

PHLPP Dephosphorylates the Hydrophobic Motif of PKC— The selectivity of PHLPP isoforms for the hydrophobic motif site of PKC is consistent with our previous report that PHLPP1 and PHLPP2 catalyze the specific dephosphorylation of the hydrophobic motif site (Ser^{473}) on a related AGC kinase, Akt (2). Previous studies have shown that a PP2A-type phosphatase associates with PKC in the membrane fraction of cells (18). Our results here suggest that the phosphorylation state of the activation loop and turn motif sites are likely controlled by a PP2A-type phosphatase because dephosphorylation of both sites is sensitive to okadaic acid treatment. In contrast, results from siRNA depletion and overexpression experiments indicate that the hydrophobic motif site (Ser^{660}) is regulated by PHLPP isoforms, consistent with the finding that the phosphorylation state of the hydrophobic motif is not sensitive to okadaic acid, an inhibitor that does not affect PP2C family members. It is noteworthy that PHLPP does not discriminate between the turn motif and hydrophobic motif *in vitro*, but selectively dephosphorylates the hydrophobic motif in cells. These data underscore the importance of cellular studies in determining substrate specificity.

In addition to conventional PKCs, the PKC family includes the novel and the atypical subclasses. Members of atypical PKCs (ζ and ι/λ) have a Glu residue instead of Ser/Thr at the phospho-acceptor position of the hydrophobic motif. Thus, this site in the atypical PKCs cannot be a substrate of PHLPP. Consistent with this, we show that the expression of PKC ζ was not altered by depletion of PHLPP from cells, whereas increased expression of PKC δ and ε was observed in the knockdown cells. These data suggest that PHLPP directly controls the levels of conventional and novel, but not atypical PKCs. Because different PKC isozymes have diverse physiological roles in regulating cellular processes (30), the functional readout of PHLPP-mediated down-regulation of PKC is likely cell context dependent.
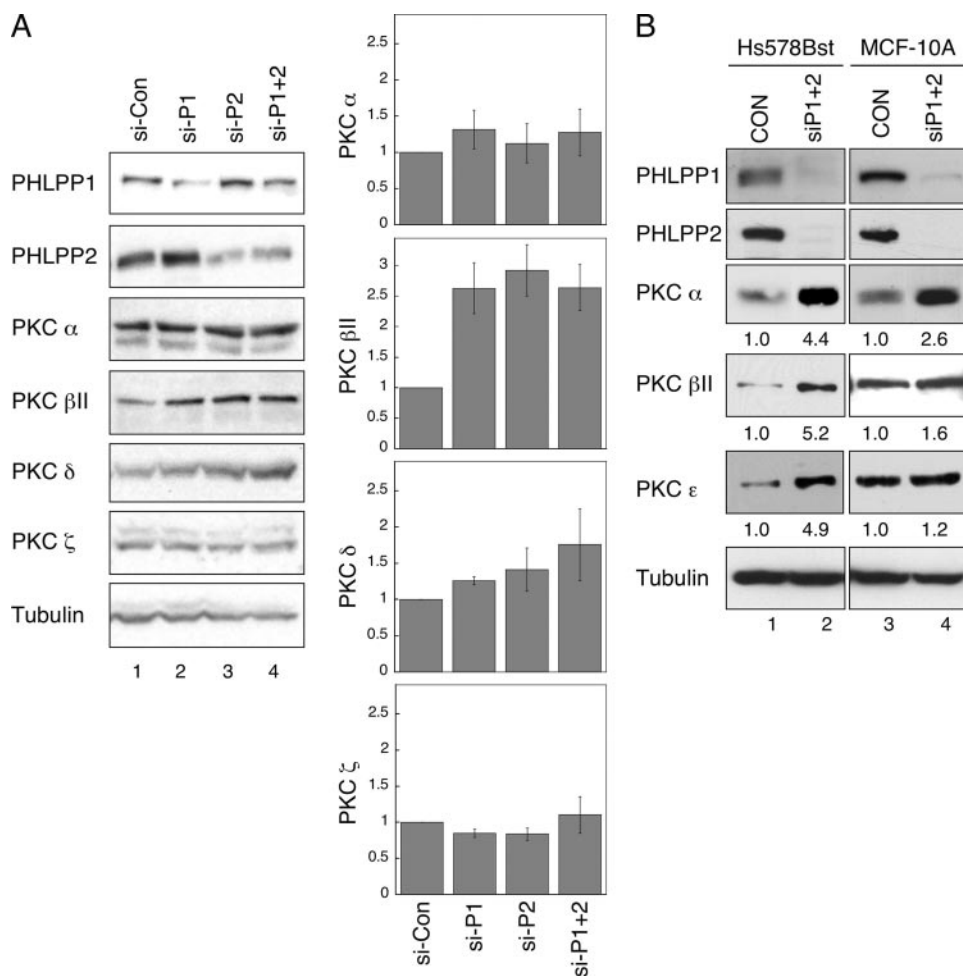
A

B

FIGURE 7. **Expression of PHLPP correlates inversely with cellular PKC levels.** *A*, knockdown of PHLPP isoforms in colon cancer DLD-1 cells results in increased expression of PKC βII. DLD-1 cells were transfected with control siRNA (*si-Con*) or siRNAs against PHLPP1 (*si-P1*), PHLPP2 (*si-P2*), or both PHLPP isoforms (*si-P1 + 2*) (*lanes 1– 4*, respectively). The whole cell lysates were analyzed by Western blot using anti-PKC α, anti-PKC βII, anti-PKC δ, or anti-PKC ζ antibodies. The *graphs* shown on the *right* are the quantitative representations of PKC expression. The relative expression level of each PKC isoform was obtained by normalizing ECL signals of specific PKC antibodies to that of tubulin. Data represent the mean ± S.E. of three independent experiments. *B*, knockdown of PHLPP isoforms in normal breast epithelial cells results in increased expression of PKC. Hs578Bst (*lanes 1* and *2*) and MCF-10A (*lanes 3* and *4*) cells were transfected with control or PHLPP-specific siRNA (a combination of siRNAs for both PHLPP1 and PHLPP2 isoforms), and whole cell lysates were analyzed by Western blot using anti-PKC α, anti-PKC βII, or anti-PKC ε antibodies. The expression of PKC relative to the control sample is indicated *below* the PKC panels.

apoptosis, and suppress tumors. In contrast, deletion of the PH domain resulted in more robust phosphatase activity toward Akt in cells (2). In striking contrast, the data presented in this study demonstrate that the PH domain of PHLPP1 is required for its ability to efficiently dephosphorylate PKC in cells, whereas the PDZ-binding motif is dispensable for this function. Indeed, deletion of the PDZ ligand increases the phosphatase activity of PHLPP1 toward PKC in cells. These data reveal that the regulatory modules of PHLPP play essential roles in driving the substrate specificity of the phosphatase and suggest that correct intracellular targeting of PHLPP is critical for specificity in its downstream signaling. Specifically, substrate access may depend on targeting the phosphatase in proximity to the substrate via scaffold proteins or by providing docking sites for the substrate directly. Consistent with this, removal of the regulatory determinants that drive Akt specificity (the PDZ-binding motif) allows more efficient dephosphorylation of PKC, whereas removal of the regulatory determinants that drive PKC specificity (the PH domain) enhances Akt dephosphorylation in cells. Thus, for example, removal of the PDZ ligand could release PHLPP from a scaffold where it localizes with Akt, increasing the pool of PHLPP available to bind PKC. The differential regulation of PKC and

Akt by the PH domain and PDZ-binding motif, respectively, provides an efficient mechanism for PHLPP to specifically dephosphorylate these two AGC family members.

Interestingly, compared with the evolutionarily conserved PDZ-binding motif in PHLPP isozymes, the PH domain was added later in evolution and is only found in mammalian PHLPP genes. This suggests that the primary function of PHLPP in lower organisms may be to antagonize Akt signaling and that the spectrum of substrates has broadened during evolution as more regulatory modules were added to the phosphatase.

PHLPP Controls Phorbol Ester-mediated Dephosphorylation of the Hydrophobic Motif—In this study, we used phorbol ester-induced dephosphorylation of conventional PKCs as a model system to study the activation-dependent down-regulation process. However, dephosphorylation of conventional PKC isozymes is not an isolated phenomenon unique to phorbol

The data from overexpression and siRNA knockdown experiments show that both members of the PHLPP family, PHLPP1 and PHLPP2, dephosphorylate conventional PKCs in cells. Similarly, both PHLPP isoforms share another substrate, Akt (2, 3). However, PHLPP1 and PHLPP2 regulate different isoforms of Akt and affect distinct signaling pathways downstream of Akt (3). It remains to be determined whether different PHLPP isoforms regulate distinct subsets of PKC-mediated signaling pathways.

Regulatory Modules Drive Substrate Specificity of PHLPP in Cells—Expression of constructs of PHLPP deleted in either the PH domain or PDZ-binding motif reveal that these regulatory modules drive substrate specificity in cells. We have shown previously that the PDZ-binding motif of PHLPP1 is necessary for the biological function of PHLPP1 toward Akt: deletion of the last three COOH-terminal residues encoding the PDZ ligand inhibits the ability of PHLPP1 to dephosphorylate Akt, promote
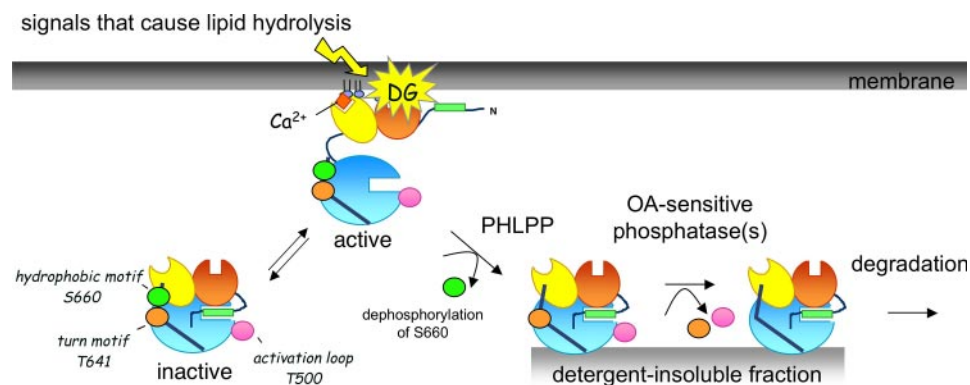
FIGURE 8. **Model showing proposed role of PHLPP in terminating the life cycle of PKC.** Mature PKC is phosphorylated at the three priming positions: the activation loop (*pink circle*; Thr^{500} in PKC βII), the turn motif (*orange circle*; Thr^{641} in PKC βII), and the hydrophobic motif (*green circle*; Ser^{660} in PKC βII). This species of PKC is maintained in an inactive state by the autoinhibitory pseudosubstrate (*green rectangle*) that occupies the substrate binding cavity in the absence of activating signals. Generation of diacylglycerol and Ca^{2+} engages PKC on the membrane by binding the C2 (*yellow*) and C1 (*orange*) domains, respectively, providing the energy to release the pseudosubstrate thus activating the kinase. This membrane-bound species of PKC has increased sensitivity to dephosphorylation. PHLPP catalyzes the first dephosphorylation event on the hydrophobic motif; this is followed by an okadaic acid-sensitive dephosphorylation of the turn motif and activation loop. Loss of phosphate at the hydrophobic motif, but not the other two sites, shunts PKC to the detergent-insoluble fraction of cells where it is eventually degraded. Note that additional down-regulation mechanisms that are independent of dephosphorylation also control PKC (15).

ester treatment. It has been reported that dephosphorylation and down-regulation of PKC occurs in cells following sustained elevation of the endogenous activator diacylglycerol (31) or when cells reach high density (14). Thus, phosphatase-mediated dephosphorylation of PKC is likely to be a general mechanism priming PKC for degradation. However, because the endogenous activators of PKC, diacylglycerol, and Ca^{2+} are only transiently elevated following agonist stimulation, only a small population of PKC may undergo dephosphorylation at a given time. The accumulation of dephosphorylated PKC is more readily observed with long lasting pharmacological treatments such as those from phorbol esters or the anti-cancer drug bryostatin (32).

Phorbol esters have been proposed to down-regulate PKC by dephosphorylation-dependent and dephosphorylation-independent mechanisms (15). Our data support a model in which PHLPP initiates the down-regulation in the dephosphorylation-dependent pathway (Fig. 8). First, we show that dephosphorylation of Ser^{660} precedes that of Thr^{641}. Second, we show that depletion of PHLPP results in the accumulation of a species of PKC that is phosphorylated on Ser^{660}, but not Thr^{641}, which is relatively resistant to degradation. Under the conditions of our experiments, ~40% of the PKC α was down-regulated by the PHLPP-dependent pathway. Fractionation studies also reveal that the trigger to send dephosphorylated PKC to the detergent-insoluble fraction is loss of phosphate at Ser^{660}.

PHLPP Controls the Cellular Levels of PKC—Elevation of PKC expression has been linked to tumorigenesis and tumor progression (27, 28, 33). However, the cause for the increased expression of PKC observed in many cancers remains unknown. In this study, we demonstrated that the level of conventional and novel PKC isozymes can be regulated by altering the expression of PHLPP1 and PHLPP2, consistent with the role of PHLPP in negatively regulating PKC by directly dephosphorylating PKC and promoting its down-regulation. We have

previously shown that the altered PHLPP levels in some of these colon cancer cell lines do not affect the expression levels of Akt, rather, cells with low PHLPP have high basal phosphorylation of the hydrophobic motif on Akt (Ser^{473}) (2). This finding underscores the markedly different effects of PHLPP dephosphorylation of the hydrophobic motif on PKC and Akt: for PKC it controls the stability and thus levels of the protein, for Akt it controls the degree of hydrophobic motif phosphorylation, and hence activity.

Consistent with a key role of PHLPP in controlling the levels of cellular PKC, the basal level of PKC expression in normal breast epithelial cells is markedly up-regulated by silencing the expression of endogenous PHLPP using RNA interference. The levels of PKC α and PKC βII increased ~5-fold in Hs578Bst cells. Note that this large increase of PKC expression upon silencing PHLPP genes observed in normal cell lines was not seen or restricted to certain PKC isozymes in cancer cell lines (or transformed cells) (see Figs. 6 and 7). It is possible that the dynamic connection linking PHLPP and PKC in the normal cells is disrupted in the cancer cells, where compensating mechanisms may be activated to confer cell survival, thus resulting in less control of PKC by PHLPP. Indeed the effects of PHLPP knockdown on agonist-evoked Akt phosphorylation are at least an order of magnitude greater in normal cells such as the Hs578Bst compared with transformed cells such as 293T (3). We note in particular that depletion of PHLPP isoforms from the DLD-1 colon cancer line caused a selective increase in PKC βII protein level. This is noteworthy because PKC βII protein levels are selectively elevated in colon cancer (27). Whether the molecular mechanism for the elevated PKC βII in colon cancer is driven by reduced levels of PHLPP remains to be explored. Further studies on the interplay between PHLPP and PKC in tumorigenesis are of potential interest in developing novel therapies in cancer treatment.

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