AD_____

Award Number: W81XWH-05-1-0275

TITLE: Regulation of Akt/protein kinase B signaling by a novel protein phosphatase in breast cancer cells

PRINCIPAL INVESTIGATOR: John Brognard, Ph.D.
Alexandra Newton, Ph.D. (Mentor)

CONTRACTING ORGANIZATION: University of California, San Diego
La Jolla, CA 92093-0602

REPORT DATE: January 2008

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
17-01-2008	Annual Summary	1 MAR 2005 - 17 DEC 2007

4. TITLE AND SUBTITLE
Regulation of Akt/protein kinase B signaling by a novel protein phosphatase in breast cancer cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0275

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
John Brognard, Ph.D.

Email: jbrognard@ucsd.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, San Diego
La Jolla, CA 92093-0602

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

See next page.

15. SUBJECT TERMS
Signal Transduction, Tumor Suppressor, Apoptosis, Cell Cycle Regulation.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	60	19b. TELEPHONE NUMBER *(include area code)*

14. ABSTRACT

The major purpose of the proposed research was to determine if a novel phosphatase, entitled PHLPP2, negatively regulates the protoncogene Akt, by dephosphorylating this kinase at a key residue, Ser 473, where phosphorylation is required formaximal activity. Furthermore, we sought to determine if this phosphatase played a role in regulating downstreamphysiological effects of Akt signaling including: cell proliferation, growth, and apoptosis. Finally, since this phosphatase resides in a location of frequent loss of heterozygosity in breast cancer, we sought to determine if this phosphatase played a role in breast tumorigenesis. Major findings, are consistent with originally proposed research, I was able to successfully clone the full length PHLPP2 protein and determine phosphatase activity in vitro and in vivo. Full length PHLPP2 is a functional phosphatase that preferentially dephosphorylates Akt at Ser 473, thereby decreasing kinase activity, promoting apoptosis, and inhibiting cellular proliferation. Consistent with these results, siRNA mediated knockdown of endogenous PHLPP2 increases phosphorylation of Akt at Ser 473, and promotes cellular proliferation and survival. Finally, we have distinguished functional roles for PHLPP1 and PHLPP2 by examining downstream signaling of the Akt kinase. PHLPP2 preferentially regulates thecell cycle inhibitor p27, while PHLPP1 regulates GSK-3α, and HDM2. Both phosphatases regulate the phosphorylationstatus of GSK-3β, TSC-2 and FoxO1. We have elucidated the molecular mechanism where PHLPP phosphatases regulateunique downstream substrates of Akt: by interacting with and dephosphorylating specific isoforms of Akt. PHLPP2 regulatesAkt1 and Akt3 while PHLPP1 regulates Akt2 and Akt3. Finally, we have discovered that the PHLPP isoforms regulate the amplitude and duration of Akt phosphorylation following agonist treatment in a normal breast cell line (Hs578Bst), further implicating this phosphatase as a major regulator of Akt in breast cells. This work has now been published in the journal Molecular Cell. We have also found that the PHLPP phosphatases regulate phosphorylation of the hydrophobic motif of PKC,resulting in regulation of PKC protein levels. While screening breast cancer cell lines for mutations a common polymorphismwas identified that resulted in an amino acid change (Leu to Ser at amino acid 1016 (L1016S)) in the PP2C domain of thephosphatase, where the a copy of the Ser allele is present in 15% of the population. Four breast cancer cell lines with LOH at16q22.3 only possess the less common Ser allele. When comparing a normal and cancer cell line from the same patient results show that the cancer cell line retains only the Ser allele. This provided us with the impetus to genotype normal and tumor tissue from the same patient, and consistent with results in breast cancer cell lines, the Leu allele is preferentially lost from high-grade breast cancer patients that are heterozygous. We then explored the role of PHLPP2 in breast cancer celllines, expression of wild type PHLPP2 in breast cancer cell lines decreases Akt phosphorylation at Ser 473 and induces apoptosis. Generation of this variant indicated that it was less functional in in vitro phosphatase assays and when expressed in cells. Finally, depletion of PHLPP2 from breast cancer cell lines that only possess the Ser Allele indicates that this polymorphic variant is less functional, but only under basal, unstimulated conditions. Surprisingly in a case control study the Ser/Ser genotype occurred at a similar frequency in breast cancer patients as age-matched controls. These results suggest that loss of the more functional Leu variant may play a role in breast cancer progression, but that possessing only the Ser allele does not predispose women to the development of breast cancer.

Table of Contents

Introduction

A prevalent underlying mechanism driving tumorigenesis is aberrant activation of signal transduction pathways resulting in increased cell growth, proliferation and/or survival (1). A well-characterized signal transduction pathway that promotes cellular survival, growth, and proliferation in breast cancer is the PI3K/Akt pathway. This pathway can be activated via a number of mechanisms including amplification or gain of function mutations in upstream receptor protein tyrosine kinases (RPTKs), constitutive activation of hormone receptors, direct amplification of PI3K or Akt, mutations in PI3K, and loss of expression of a regulatory phosphatase, such as PTEN (phosphatase and tensin homolog on chromosome ten)(2). PTEN mutations and deletions have been identified in a majority of cancers, however mutations are not as common in breast cancer and occur in only 6% (4/65) of breast cancer cell lines or xenografts, and are very infrequent in primary breast specimens (3-5). Since PTEN mutations are rare in breast cancer this suggests that another phosphatase that regulates Akt activity may be mutated or have loss of expression in breast cancer. My lab recently discovered a novel phosphatase PHLPP, for PH domain Leucine-rich repeat Protein Phosphatase, which regulates Akt signaling by directly dephosphorylating Akt at the hydrophobic motif, Ser 473 (6). The focus of my thesis work was to identify, clone, and characterize a second isoform of the PHLPP family of phosphatases entitled PHLPP2. Based on the function and location of PHLPP2 (at chromosome 16q22.3, a region of frequent loss of heterozygosity in breast cancer) we seek to determine if this protein could be a potential tumor suppressor in breast cancer.

Body

Functional Characterization of the PHLPP2 phosphatase

We were able to successfully clone the full length PHLPP2 and details of the exact procedures used are included in the manuscript in the appendix of this annual report. Results demonstrated that the full length PHLPP2 preferentially dephosphorylates Akt at Ser 473, unique from the isolated PP2C domain of PHLPP2, which dephosphorylates both Ser 473 and Thr 308 of Akt (7). Consistent with these results, expression of PHLPP2 in cells results in a decrease in Akt phosphorylation at Ser 473, with no effects on phosphorylation at Thr 308 (7). This decrease in phosphorylation of Akt at Ser 473 correlated with a decrease in kinase activity (7). We observed the PHLPP2 phosphatase to be localized in the cytosol and nucleus (7) and this was consistent with overexpression results (unpublished data). Finally we observed that the PH domain of PHLPP1 and PHLPP2 has similar specificity for phosphorylated phosphoinositides, which included PI3P, PI4P, PI3,5P and PI3,4,5P (unpublished results). This concluded the goals of task 1 in the SOW. To verify the role of endogenous PHLPP2 we specifically knocked down PHLPP2 and this resulted in increase in phosphorylation of Akt at Ser 473 (7). We monitored depletion of PHLPP2 expression using isoform specific antibodies for the PHLPP2 phosphatase that do not cross-react with PHLPP1 (7). Co-immunoprecipitation experiments indicated that Akt and PHLPP2 associate in cells (7). Finally, the PHLPP isoforms regulate both the amplitude and duration of Akt phosphorylation. Loss of either PHLPP1 or PHLPP2 in a normal breast cell line (Hs578Bst) that was serum starved and treated with EGF results in dramatic 30-fold increase in the amplitude of Ser 473 phosphorylation of Akt as well as prolonged phosphorylation. This strongly implicates the importance of the PHLPP phosphatases in regulating Akt in normal breast cells and how loss of this phosphatase could result in constitutive activation of the Akt kinase, which could ultimately lead to the tumorigenic phenotype. We also observed a dramatic increase in T308 phosphorylation suggesting the phosphatases may be regulating this site as well under agonist-stimulated conditions. An alternative explanation for the increase in T308 phosphorylation is that hyperphosphorylated Akt at Ser 473 may make the kinase more resistant to phosphatases or a better substrate for PDK-1. Depletion of PHLPP1 or PHLPP2 from sin1 null cells, which lack Ser 473 phosphorylation, did not result in a robust increase in T308 phosphorylation, confirming our hypothesis (data not shown). We did observe modest increases in both S473 and T308 phosphorylation in sin1 null cells. Many of the above findings were crucial to our understanding of the PHLPP2 phosphatase, but were not included in the SOW.

Akt is a pivotal regulator of cell growth, proliferation and survival. To assess whether negative regulation of this kinase by PHLPP2 altered any of these cellular functions we monitored apoptosis and

cell cycle progression. To test whether PHLPP2 regulated apoptosis we selected a cell line that expressed hyperphosphorylated Akt and had previously been to shown to be sensitive to Akt inhibition (8). PHLPP2 expression in these cells caused an increase in apoptosis, under conditions of serum deprivation (7). Similar results were observed in breast cancer cell lines, however we were unable to identify any breast cancer cell lines that lacked expression of PHLPP2 (Figure 4). Depletion of PHLPP2 from cells decreased apoptosis and protected cells from etoposide induced apoptosis, suggesting that loss of the PHLPP phosphatases could play a role chemotherapeutic resistance (7). Expression of PHLPP2 under high serum conditions caused an increase in G1/S ratio suggesting PHLPP2 is inhibiting cell cycle progression. Consistent with these results, knockdown of PHLPP2 decreased the G1/S ratio and increased BrdU incorporation in a normal breast cell line confirming the role of PHLPP2 as a negative regulator of the cell cycle (7). These results concluded all research aims included in Task 3 of the SOW. Surprisingly, reducing PHLPP1 levels did not decrease the G1/S ratio or increase proliferation as dramatically as PHLPP2 knock down. To address this discrepancy we examined the effects of knocking down PHLPP1, PHLPP2, or both on specific downstream targets of Akt. Knockdown studies reveal that PHLPP1 specifically controls the activity of Akt towards the substrates HDM2 and GSK-3α, whereas PHLPP2 specifically controls the activity of Akt towards the cell cycle inhibitor p27 (7). To address the mechanism driving the differential downstream substrate regulation we examined whether the PHLPP phosphatases were binding and regulating distinct Akt isoforms. We discovered that PHLPP2 is regulating Akt1 and Akt3 while PHLPP1 regulates Akt2 and Akt3 (7). To verify that these Akt isoforms are regulating the substrates that are differentially regulated by the PHLPP isoforms we knocked down Akt isoforms singularly and examined the phosphorylation status of downstream substrates. These data revealed that PHLPP1 is regulating Akt2 to regulate the phosphorylation of HDM2 and GSK-3α. Surprisingly, PHLPP2 is regulating Akt3, which in turn regulates p27 phosphorylation status suggesting a novel mechanism of cellular localization must be involved in this regulation since both PHLPP isoforms regulate Akt3. Both PHLPP isoforms regulate FoxO1 and GSK-3β phosphorylation by regulating their specific Akt isoforms. Finally PHLPP2 regulates Akt1 to regulate the phosphorylation status of TSC2 while PHLPP1 regulates Akt2, which regulates the phosphorylation status of TSC2 (7). As a test of this molecular circuitry we depleted a normal breast cell line (Hs578Bst) of Akt3, PHLPP2 or both stimulated cells with EGF and determined if loss of Akt3 would inhibit PHLPP2 regulation of p27. Indeed, loss of both PHLPP2 and Akt3 blocked the increase in p27 phosphorylation observed when only PHLPP2 is depleted (7). This research fulfills the aims of task 4 as we have elucidated the PHLPP2 phosphatase as a novel regulator of Akt downstream substrate p27. We did not examine cellular localization of p27 since phosphorylation should result in cytosolic sequestration. We did not observe changes in p21 phosphorylation (data not shown). Additionally, overexpression of PHLPP1 or PHLPP2 results in loss of specificity for unique Akt isoforms, thus overexpression of PHLPP2 results in a global decrease in phosphorylation status of Akt substrates (7).

During these studies we observed that loss of both PHLPP isoforms did not result in an additive increase in Akt phosphorylation at Ser 473, inconsistent with the PHLPP isoforms regulating unique Akt isoforms. To address the molecular mechanism at the root of this discrepancy we tested whether the PHLPP phosphatases may be activating the p70S6K, which controls a negative feedback loop resulting in a decrease in Akt phosphorylation. Consistent with this hypothesis we observed an increase in p70S6K phosphorylation, which correlates with an increase in activity, when both PHLPP isoforms are knocked down (7). Treatment of cells with rapamycin, which inhibits p70S6K and shuts down this negative feedback loop, results in an additive increase in Ser 473 phosphorylation when both PHLPP isoforms are depleted from cells (7). Thus the depletion of both PHLPP isoforms results in activation of p70S6K, which in turn activates a negative feedback loop decreasing Akt phosphorylation and accounts for the absence of an additive increase in Akt phosphorylation when both PHLPPs are depleted from cells. This research was not included in the SOW, but was important to further our understanding of how PHLPPs regulate Akt phosphorylation at the hydrophobic motif.

This data sets the stage for understanding how the PHLPP isoforms contribute to regulation of Akt downstream substrates and was reviewed in the same issue of Molecular Cell that it was published (9).

PHLPP2 Regulates PKC Protein Levels

Identification of novel substrates of the PHLPP phosphatases should help us to understand how these phosphatases regulate proliferation and cellular survival. We have identified the novel substrate PKC, which is a member of the AGC family of kinases (10). We observe that PHLPP dephosphorylates PKC directly at the hydrophobic motif, and this leads to decreased stability and degradation of members of PKC family of kinases (10). Endogenous PHLPPs play important role in setting the level of PKC kinases expressed in cells as depletion of these phosphatases results in an increase in PKC expression (10). This research was not included in the SOW, however it is extremely important to identify other novel targets of the PHLPP phosphatases that could play a role in the process of tumorigenesis and this grant was cited in the publication.

PHLPP2 as a Potential Tumor Suppressor in Breast Cancer

In an effort to determine if the PHLPP2 phosphatase may be mutated in breast cancer we sequenced RNA from 18 breast cancer cell lines. We discovered a T->C nucleotide change at position 3047 that results in a amino acid change from Leu to a Ser at codon 1016 in the PP2C phosphatase domain. We observed that four breast cancer cell lines possessed only the Ser allele, one breast cancer cell line possessed both Ser and Leu allele, and a majority of breast cancer cell lines possessed only the Leu allele. The presence of both alleles in the MCF-7 cell line suggested this nucleotide change could potentially be a polymorphism. To determine if this was a polymorphism we genotyped 100 DNA samples from a control population from the Coriell Institute (NA17201-17300). We observed the Leu/Leu genotype in 70 individuals and the Leu/Ser genotype in 30 individuals. We never observed the Ser/Ser genotype in the control population. Additionally, we genotyped 33 breast tumor tissue samples and observed the Leu/Leu genotype in 25 patients and the Leu/Ser genotype in 8 patients, again we did not observe the Ser/Ser genotype. This research concludes task 2 of the SOW since we identified this nucleotide alteration that originally thought was a somatic mutation. In addition we did not observe any loss of expression of PHLPP2 protein or RNA levels (Figure 4 and data not shown).

The observation of only the Ser allele in breast cancer cells, but not in the control population suggested this polymorphism may contribute to the breast cancer phenotype. Since one of the most common genetic lesions in breast cancer is loss of heterozygosity on chromosome 16q22, in the chromosomal location where PHLPP2 is present, we sought to determine if the presence of only the Ser allele in breast cancer cell lines may be due to loss of heterozygosity. To address this question we genotyped the normal breast cell line (Hs578Bst) that is from the same patient as the breast cancer cell line Hs578t. We observed the genotype of the normal cell line to be Leu/Ser, therefore the breast cancer cells from that patient lost the Leu allele and retained only the Ser allele (Figure 1). We cannot determine if loss of heterozygosity is the mechanism underlying the presence of the only Ser allele in the remaining breast cancer cell lines since no normal breast cell lines from the same patient exist for these cancer cell lines, however LOH at 16q has been reported for this chromosomal region in these cell lines (11). To note, in the one breast cancer cell line that possessed both alleles, the MCF-7 cell line, the Leu allele is consistently expressed at lower levels based on sequencing suggesting this allele could be methylated.

We next sought to ascertain the phosphorylation status of the hydrophobic motif of Akt in the Hs578t cell line compared to the normal Hs578Bst cell line since the PHLPP2 phosphatase regulates phosphorylation at this site. Comparison of the two cell lines showed that the tumor cell line had a dramatic increase in Ser 473 phosphorylation compared to the normal cell line (Figure 1). Given that the Hs578t breast cancer cell line does not have mutations in the PTEN tumor suppressor or the PIK3CA oncogene (10), we hypothesized that the PHLPP2 variant with a ser at position 1016 may be less functional and contribute to the hyper-phosphorylation of Akt in these cells.

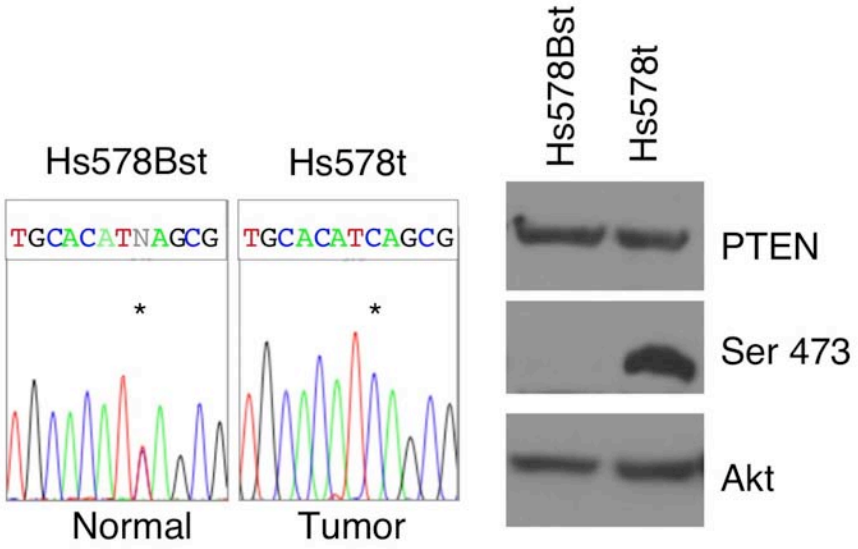

Figure 1 Comparison of a breast cancer cell line and normal cell line from the same patient shows the cancer cells have lost the Leu allele. Constitutive activation of Akt in the cancer cell line compared to the normal cell line despite WT PTEN and PIK3CA.

Characterization of the Leu1016Ser Substitution

To determine if Ser at codon 1016 alters phosphatase activity we substituted a Ser for a Leu in the full length PHLPP2 and observed decreased phosphatase activity toward Akt at Ser 473 in an *in vitro* dephosphorylation assay when comparing it to the Leu containing full-length PHLPP2, Figure 2. These data suggest that this genetic alteration correlates with decreased activity of the PHLPP2 phosphatase and this could contribute to increased Akt phosphorylation and tumor progression.

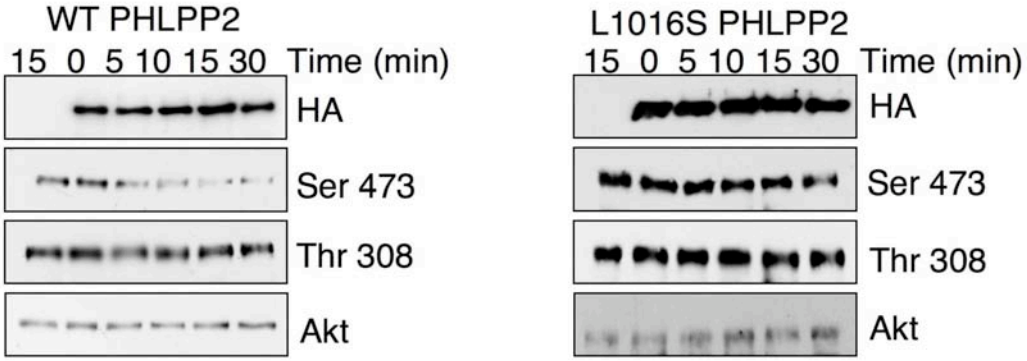

Figure 2 Full length L1016S shows less activity towards fully phosphorylated Akt than PHLPP2 with Leu at position 1016.

To further characterize the L1016S substitution we expressed both variants in cells and monitored the phosphorylation status of Akt. When full length PHLPP2 containing a Leu at position 1016 is expressed in the H157 cells we observed a decrease in phosphorylation of Akt at Ser 473 consistent with previous results, Figure 3, Lane 2. Strikingly, we observed only modest effects on Ser 473 phosphorylation in cells expressing PHLPP2 with a Ser at position 1016, Figure 3. These results are consistent with phosphatase assay results, where substitution of a Leu for a Ser results in a dramatic decrease in PHLPP2 catalytic activity. To determine if this would alter cellular processes PHLPP2 has been demonstrated to regulate, we expressed both variants in the H157 cells and monitored apoptosis. Figure 3 shows that Ser containing PHLPP2 attenuates the apoptotic response: expression of PHLPP2 (Leu) increases apoptosis 5-fold while the L1016S only increases apoptosis 2-fold. These results imply

that the loss of catalytic activity of the L1016S decreases the regulation of phosphorylation of Akt and suppresses the apoptotic phenotype.

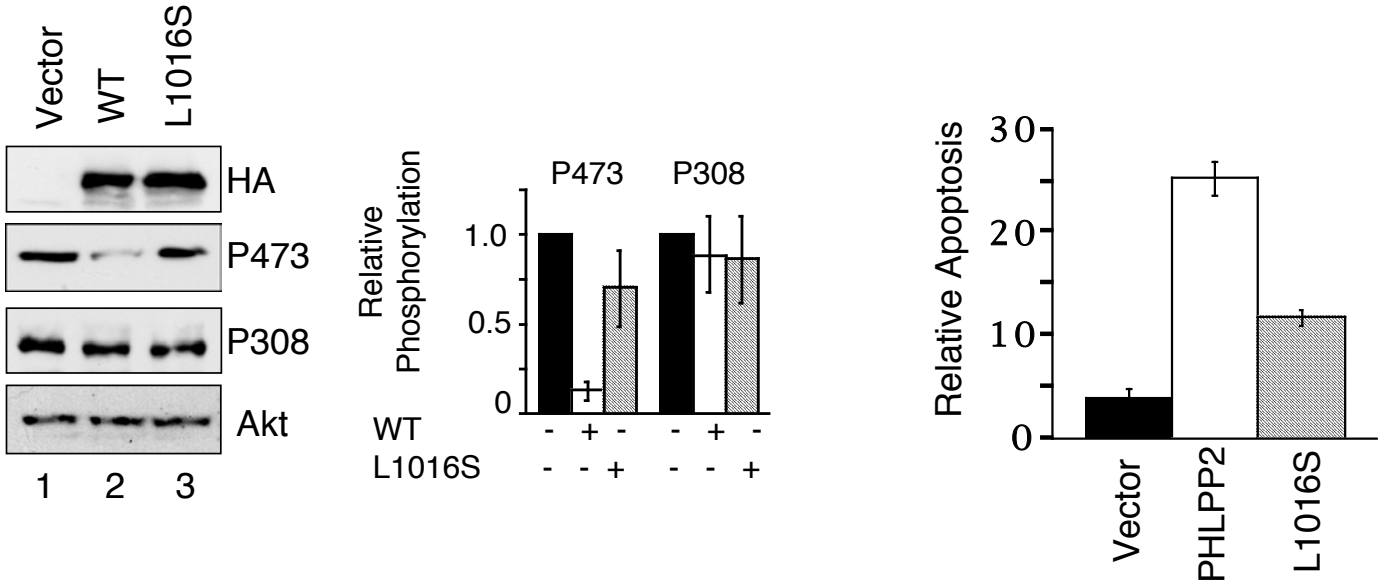

Figure 3. The L1016S PHLPP2 variant shows decreased phosphatase activity towards Akt and reduced induction of apoptosis.

Evaluating the Functionality of Endogenously Expressed L1016S

To determine if the Leu to Ser substitution would alter the function of the endogenously expressed L1016S we depleted the PHLPP2 variants in breast cancer cell lines that possessed only the Ser allele, both alleles or only the Leu allele and monitored the phosphorylation status of Akt and protein levels of PKC. To note the Leu to Ser change did not alter expression levels of the PHLPP2 phosphatase. Consistent with overexpression studies depletion of endogenous Ser variant did not alter phosphorylation status of Akt at Ser 473 in breast cancer cell lines, Figure 4. When comparing the normal breast cancer cell line, Hs578Bst, to the pair-matched breast cancer cell line, Hs578t, data indicate that only one functional Leu allele of PHLPP2 is required to regulate Akt phosphorylation at Ser 473. For comparison depletion of PHLPP2 from cell lines, which possesses only the Leu allele results in an increase in Ser 473 phosphorylation and an increase in PKC protein levels, suggesting these cell lines still possesses a functional PHLPP2. Interestingly, depletion of PHLPP1 in the Hs578t cell line does not result in an increase in Ser 473 phosphorylation, suggesting this cell line may possess a mutated and non-functional PHLPP1 phosphatase. Somatic mutations in the PHLPP1 phosphatase will be the focus of a future study.

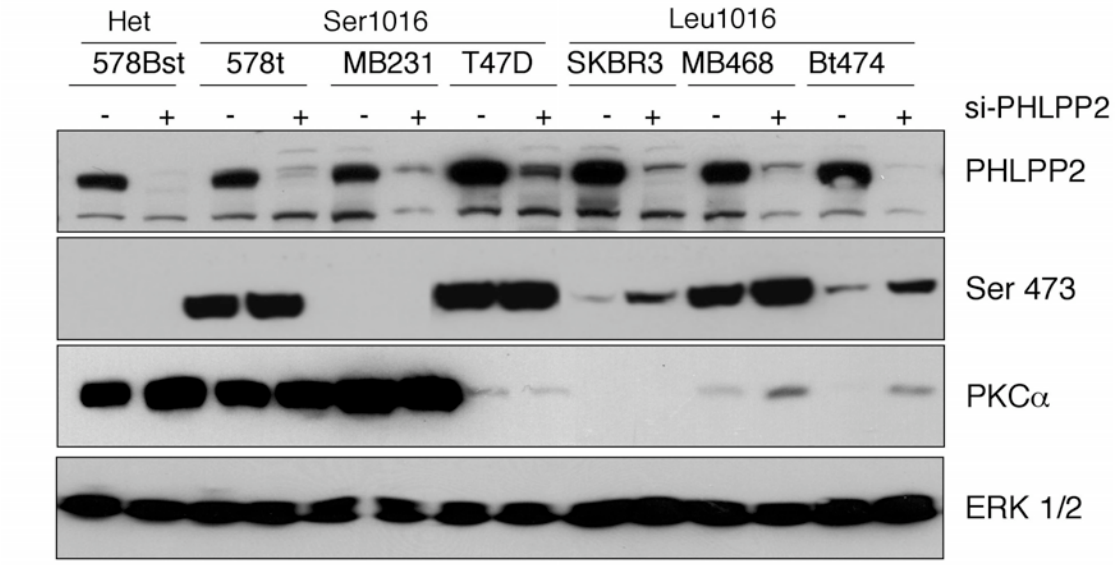

Figure 4 Depletion of endogenous PHLPP2 from breast cancer cells that only possess the Ser allele shows that this polymorphic variant is less functional than Leu containing PHLPP2.

We previously reported that the PHLPP family of phosphatases regulate both the amplitude and duration of agonist-induced Akt activation. Thus, we next asked whether the Ser variant is less functional at regulating agonist-induced phosphorylation of Akt. We tested the effect of PHLPP2 knockdown on agonist-induced activation of Akt in breast cancer cell lines expressing only the Ser1016 variant. The Western blots in Figure 5 reveal that depletion of PHLPP2 in the Hs578t, T47D, MB231, or ZR-75-1 cells resulted in a dramatic increase in both the amplitude and duration of Akt phosphorylation. In fact, the magnitude of the knockdown effects were on the same order of magnitude as that observed in the normal breast cell line Hs578Bst or Bt474 breast cancer cell line expressing only the Leu allele when compared to the T47D, MB231 or ZR-75-1 cell lines. These data reveal that the Ser1016 variant is less functional at controlling Akt under basal or serum starvation conditions in all breast cancer cells, but it effectively suppresses agonist-evoked phosphorylation of Akt.

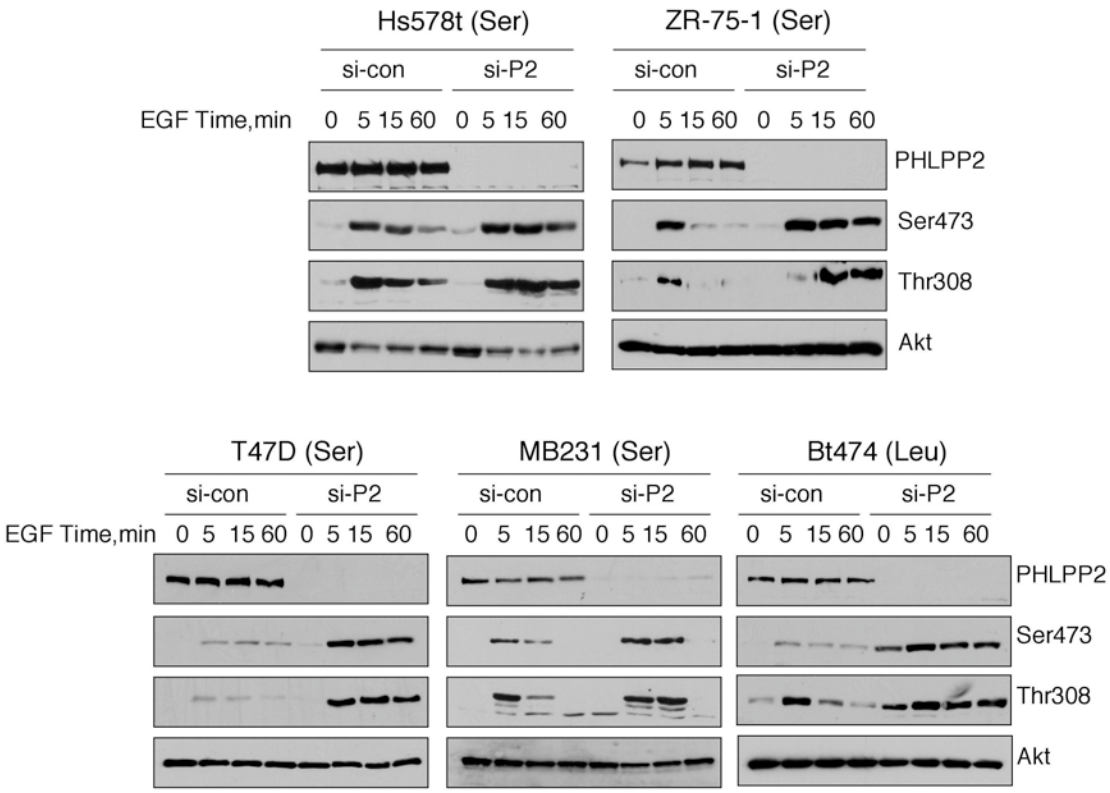

Figure 5 Ser variant of PHLPP2 remains functional under agonist-stimulated conditions.

Loss of the Leu Allele from High-Grade Breast Cancers

To determine if the Leu allele is lost in breast cancer tumor tissue compared to normal tissue from the same patient, similar to that observed in the Hs578Bst-Hs578t cell lines, we genotyped pair-matched normal and tumor tissue samples. Of the 33 samples we genotyped 8 possessed the polymorphism, of these eight 5 were grade 2 ductal breast carcinomas and 3 were grade 3 ductal breast carcinomas. Surprisingly all 3 grade 3 tumor tissue samples exhibited loss of the Leu allele, however loss of either allele was not observed in the 5 grade 2 breast tumor tissue samples (Figure 6). These data suggest that preferential loss of the Leu allele in high-grade breast cancer may contribute to the aggressive phenotype of these cancers.

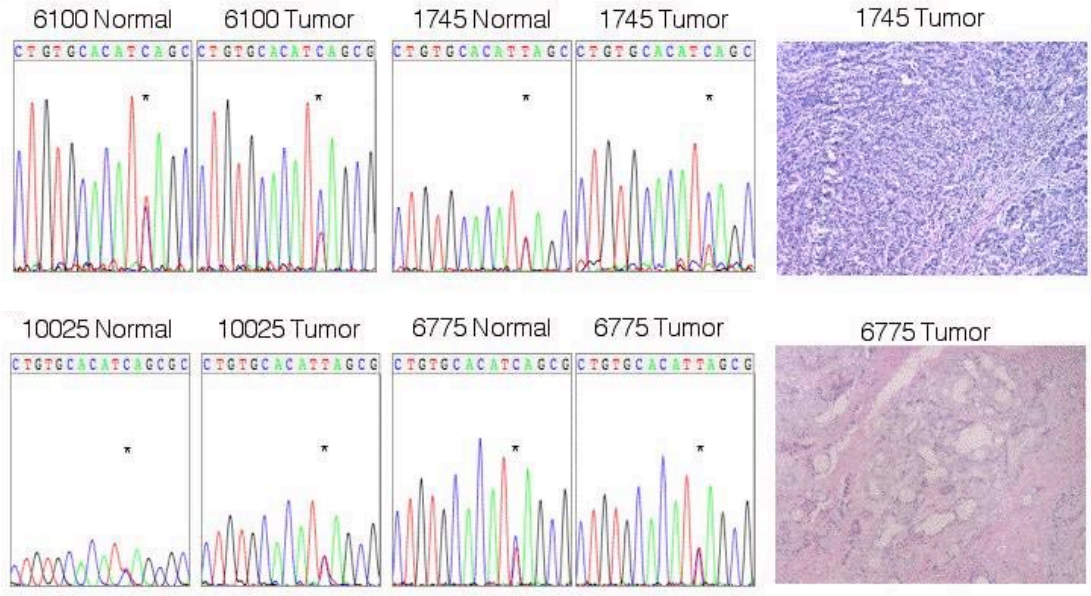

Figure 6 Loss of the Leu allele in high-grade breast cancers, but not low-grade breast cancers.

Examination of PHLPP2 variant allele frequencies in breast cancer patients

To determine if women with the PHLPP2 polymorphism are predisposed to the development of breast cancer, we genotyped 618 invasive breast cancers, 96 *in situ* breast cancers, and 583 population controls (representative chromatograms displayed in Figure 5D). Approximately 15% of controls carried the variant Ser allele. Carriers were at no greater risk of breast cancer when compared to noncarriers (age- and study site-adjusted odds ratio (OR): 1.01; 95% confidence interval (CI): 0.79-1.30; p=0.93). However, carriers of two variant alleles had a nonsignificantly reduced breast cancer risk (age- and site-adjusted OR: 0.56; 95% CI: 0.25, 1.26; p=0.16) (Table 1). This genotype was rare in the population (2.7% among controls).

Table 1. Risk of breast cancer according to the L1016S PHLPP2 genotype in a US population-based case-control study

	Invasive Breast Cancer			In Situ Breast Cancer			All Breast Cancers		
Genotype	Case (618)	Ctrl (559)	OR (95% CI)	Case (96)	Ctrl (583)	OR (95% CI)	Case (714)	Ctrl (583)	OR (95% CI)
LEU/LEU	449	408	1.00 (ref)	67	425	1.00 (ref)	516	425	1.00 (ref)
LEU/SER	159	136	1.04 (0.79,1.36)	29	142	1.08 (0.65,1.78)	188	142	1.06 (0.82,1.37)
SER/SER	10	15	0.67 (0.29,1.53)	0	16	na	10	16	0.56 (0.25,1.26)

Key Research Accomplishments

- Demonstrated that the PHLPP phosphatases regulate both amplitude and duration of agonist induced activation of Akt.
- Provided evidence that both PHLPP isoforms inhibit apoptosis and protect cells against etoposide-induced apoptosis suggesting a novel role for the phosphatases in chemotherapeutic resistance. This was determined using PHLPP isoform specific siRNA.
- Discovered that the PHLPP phosphatases interact with and regulate distinct Akt isoforms.
- We have discovered by use of siRNA that PHLPP2 regulates Akt1 activity towards the downstream substrate TSC-2 and Akt3 activity towards the downstream substrate p27. We have observed that PHLPP1 regulates Akt2 activity towards downstream substrates HDM2, GSK-3α, and TSC-2. Finally, we have observed that both PHLPP1 and PHLPP2 regulate phosphorylation status of GSK-3β and FoxO1 by regulating activities of Akt2 and Akt3 or Akt1 and Akt3, respectively.
- We observed that this distinct Akt isoform regulation also occurs under growth factor conditions, where PHLPP2 loss results in an increase in p27 phosphorylation when cells are stimulated with EGF, and this increase in phosphorylation is lost when Akt3 is depleted from cells.
- We uncovered a novel mechanism of how loss of both PHLPP phosphatases does not result in an additive increase in Akt phosphorylation at Ser473. Loss of both PHLPP isoforms increases

p70S6K phosphorylation resulting in activation of a negative feedback loop that decreases Ser 473 phosphorylation. Inhibition of p70S6K activation with the drug rapamycin shuts down this feedback loop and results in an additive increase in Ser 473 phosphoryltion when the PHLPP phosphatases are depleted from cells.

- We have Identified a novel polymorphism in PHLPP2. This polymorphism results in an amino acid change from a Leucine to a Serine in the PP2C domain. Frequency of the polymorphism in the population is 30%. Only the Ser allele was detected in four breast cancer cell lines. One of the four breast cancer cell lines, had a normal cell line generated from normal tissue of the same patient. Loss of the more prevalent Leu allele was observed. Finally, high-grade breast cancer tissues have decreased expression of Leu allele when compared to normal tissues from the same patient. This suggests loss of the more functional Leu allele may promote tumor progression.

- Generated the variant PHLPP2, which has a serine at amino acid 1016 and is referred to as L1016S. The L1016S has less catalytic activity compared to Leu containing PHLPP2 in *in vitro* phosphatase assays and when expressed in cells. Consistent with this expression of the L1016S attenuates the apoptotic response compared to the Leu containing PHLPP2.

- We have observed that loss of endogenous PHLPP2 from cells that only possess the Ser allele for PHLPP2 does not increase Akt phosphorylation at Ser473 or PKC protein levels under high serum conditions, consistent with this variant being less functional than the more prevalent Leu containing PHLPP2.

- Interestingly the Ser variant seems to retain function, or overcome inhibtion under agonist-induced conditions, and thus is quite functional at regulating Akt under these conditions.

- Women that are homozygous for the Ser allele are not at an increased risk for the development of breast cancer, likely due to compensatory mechanisms, since these women developed with the less functional allele.

Reportable Outcomes

- Manuscript published in Molecular Cell (appendix), and a review was also written regarding this manuscript in the same issue of Molecular Cell (3/07).
- Manuscript published in JBC (appendix), I am second author.
- Polymorphism manuscript to be submitted to Cancer Research
- Review of PHLPP Phosphatase, submitted, Trends in Endocrinology and Metabolism
- Poster Presentation, Keystone Symposia - Molecular Targets for Cancer
- Poster Presentation, AACR Forefront of Basic and Translational Cancer Research meeting (1/07)
- Oral Presentation, Salk Institute 12th Meeting on Protein Phosphorylation and Cell Signaling (8/06)
- Oral Presentation, PTEN Pathways Meeting, Cold Spring Harbor Meeting (3/06)

Conclusions

In conclusion we have successfully cloned a novel phosphatase, PHLPP2, and demonstrated that this is a functional phosphatase targeting Akt *in vitro* and *in vivo*. We have shown that PHLPP2 directly dephosphorylates Akt at the hydrophobic motif, Ser 473, and this results in a decrease Akt kinase activity. This decrease in kinase activity correlates with an increase in apoptosis, under conditions of serum deprivation, and inhibition of cell cycle progression, under high serum conditions. Consistent with these results depletion of endogenous PHLPP2 increases phosphorylation of Akt at Ser 473, and

increases cellular proliferation and survival. Further characterizing agonist induced activation of Akt, we discovered that the PHLPP isoforms regulate the amplitude and duration of Akt activation. When comparing PHLPP1 and PHLPP2 knockdown studies reveal that PHLPP1 specifically controls the activity of Akt towards the substrates HDM2 and GSK-3α, whereas PHLPP2 specifically controls the activity of Akt towards the cell cycle inhibitor p27. This is through direct regulation of distinct Akt isoforms, where PHLPP2 regulates signaling through Akt1 and Akt3, while PHLPP1 regulates signaling by Akt2 and Akt3. This unique isoform regulation is the molecular mechanism responsible for the PHLPP isoforms regulating distinct downstream substrates of Akt and this was verified by depleting cells singularly of each Akt isoform.

We have identified a nonsynonymous polymorphism in the PHLPP2 phosphatase that results in substitution of a Ser for a Leu at position 1016 in the PP2C domain. Biochemical analysis reveals that the less common variant, Ser1016, has impaired phosphatase activity towards the substrate Akt *in vitro*. Similarly, overexpression studies reveal that the Ser1016 variant is less effective at dephosphorylating Akt in cells, and thus less effective at inducing apoptosis compared to the Leu1016 variant. Consistent with impaired biological function, genetic depletion of PHLPP2 in unstimulated (but not EGF-stimulated) cells expressing only the Ser1016 allele does not significantly affect either basal Akt phosphorylation or total PKC levels, whereas depletion of PHLPP2 in cells expressing the Leu1016 allele results in an increase in Akt phosphorylation and total PKC levels. We observe specific loss of the Leu1016 variant from a breast cancer cell line and high-grade breast cancer tumor tissue samples when compared to controls suggesting loss of the more functional variant may contribute to tumor progression. However, there is not an increased risk for the development of breast cancer in carriers of the Ser allele. These data highlight the complexity of the role of these PHLPP2 variants in breast cancer.

References

1. Blume-Jensen P, Hunter T. Oncogenic kinase signalling. Nature. 2001 May 17;411:355-65
2. Shen Q, Brown PH. Novel agents for the prevention of breast cancer: targeting transcription factors and signal transduction pathways. J Mammary Gland Biol Neoplasia. 2003 Jan;8:45-73.
3. Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science. 1997 Mar 28;275:1943-7.
4. Freihoff D, Kempe A, Beste B, Wappenschmidt B, Kreyer E, Hayashi Y, Meindl A, Krebs D, Wiestler OD, von Deimling A, Schmutzler RK. Exclusion of a major role for the PTEN tumour-suppressor gene in breast carcinomas. Br J Cancer. 1999 Feb;79(5-6):754-8.
5. Ueda K, Nishijima M, Inui H, Watatani M, Yayoi E, Okamura J, Yasutomi M, Nakamura Y, Miyoshi Y. Infrequent mutations in the PTEN/MMAC1 gene among primary breast cancers. Jpn J Cancer Res. 1998 Jan;89:17-21.
6. Gao T, Furnari F, Newton AC. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell. 2005 Apr 1:13-24.
7. Brognard J, Sierecki E, Gao T, Newton AC. PHLPP and a second isoform, PHLPP2, differentially attenuate the amplitude of Akt signaling by regulating distinct Akt isoforms. Molecular Cell. 2007 Mar 23;25:917-31
8. Brognard J, Clark AS, Ni Y, Dennis PA. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res. 2001 May 15:3986-97.
9. Mendoza MC, Blenis J.PHLPPing it off: phosphatases get in the Akt. Mol Cell. 2007 Mar 23:798-800.
10. Gao T, **Brognard J**, Newton AC. The Phosphatase PHLPP Controls the Down-Regulation of Protein Kinase C. J Biol Chem. 2007 Dec 27; [Epub ahead of print].

11. Callen DF, Crawford J, Derwas C, Cleton-Jansen AM, Cornelisse CJ, Baker E. Defining regions of loss of heterozygosity of 16q in breast cancer cell lines. Cancer Genet Cytogenet. 2002 Feb:76-82

References for Reportable outcomes:

Manuscripts:

Brognard J, Sierecki E, Gao T, Newton AC. PHLPP and a New Isoform, PHLPP2, Differentially Attenuate the Amplitude of Akt Signaling by Regulating Distinct Akt Isoforms. Mol Cell. 2007 Mar 23:917-31.

Gao T, **Brognard J**, Newton AC. The Phosphatase PHLPP Controls the Down-Regulation of Protein Kinase C. J Biol Chem. 2007 Dec 27; [Epub ahead of print].

Brognard J, Newton AC. PHLPPing the switch in growth factor signaling: role of the novel family of PHLPP phosphatases in inactivating Akt. Manuscript submitted, Trends in Endocrinology and Metabolism.

Brognard J, Niederst M, Sierecki E, Newton AC. A Nonsynonymous Polymorphism in the PHLPP2 Phosphatase, which Negatively Regulates Akt Signaling, Results in Loss of Function and is Preferentially Retained in Breast Cancer. Manuscript in Preparation.

Mendoza MC, Blenis J.PHLPPing it off: phosphatases get in the Akt. Mol Cell. 2007 Mar 23:798-800.

Oral Presentations:

Brognard J, Gao T, Newton AC. Isoform Specific Regulation of Akt Signaling by the PHLPP Family of Phosphatases. Salk Instititue 12th Meeting on Protein Phosphorylation and Cell Signaling. August 18th – 22nd, 2006.

Brognard J, Gao T, Newton AC. Differential Termination of Akt Signalling by a Second Isoform of the Novel Phosphatase PHLPP. PTEN Pathways Meeting, Cold Spring Harbor. March 15th-19th 2006.

Poster Presentations:

Brognard J, Niederst M, Sierecki E, Newton AC. Identification of a Functional Polymorphism in the Phosphatase Domain of PHLPP2 that is Preferentially Retained in High Grade Breast Cancer. In the Forefront of Basic and Translational Cancer Research. AACR/JCA 7th Joint Conference, Jan. 21st-25th, 2007.

Brognard J, Niederst M, Sierecki E, Newton AC. Identification of a Functional Polymorphism in the Phosphatase Domain of PHLPP2 that is Preferentially Retained in High Grade Breast Cancer. Molecular Targets of Cancer. Keystone Symposia. March 18th-23rd 2007.

Molecular Cell
Article

PHLPP and a Second Isoform, PHLPP2, Differentially Attenuate the Amplitude of Akt Signaling by Regulating Distinct Akt Isoforms

John Brognard,[1,2] Emma Sierecki,[1] Tianyan Gao,[1,3] and Alexandra C. Newton[1,*]
[1] Department of Pharmacology
[2] Biomedical Sciences Graduate Program
University of California at San Diego, La Jolla, CA 92093, USA
[3] Present address: Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX 77555, USA.
*Correspondence: anewton@ucsd.edu
DOI 10.1016/j.molcel.2007.02.017

SUMMARY

Akt/protein kinase B controls cell growth, proliferation, and survival. We recently discovered a novel phosphatase PHLPP, for *P*H domain *l*eucine-rich repeat *p*rotein *p*hosphatase, which terminates Akt signaling by directly dephosphorylating and inactivating Akt. Here we describe a second family member, PHLPP2, which also inactivates Akt, inhibits cell-cycle progression, and promotes apoptosis. These phosphatases control the amplitude of Akt signaling: depletion of either isoform increases the magnitude of agonist-evoked Akt phosphorylation by almost two orders of magnitude. Although PHLPP1 and PHLPP2 both dephosphorylate the same residue (hydrophobic phosphorylation motif) on Akt, they differentially terminate Akt signaling by regulating distinct Akt isoforms. Knockdown studies reveal that PHLPP1 specifically modulates the phosphorylation of HDM2 and GSK-3α through Akt2, whereas PHLPP2 specifically modulates the phosphorylation of p27 through Akt3. Our data unveil a mechanism to selectively terminate Akt-signaling pathways through the differential inactivation of specific Akt isoforms by specific PHLPP isoforms.

INTRODUCTION

Akt controls the exquisite balance between cell survival and apoptosis, as well as proliferation and quiescence. Signaling molecules that tip the balance toward survival, growth, and proliferation typically bind receptors that activate the lipid kinase phosphatidylinositol 3-kinase (PI3K), resulting in production of 3′-phosphoinositide lipid second messengers, most notably phosphatidylinositol-3,4,5-trisphosphate (PtdIns P3) (Altomare and Testa, 2005; Datta et al., 1999; Vivanco and Sawyers, 2002). This lipid recruits Akt to the plasma membrane, where Akt is activated by two sequential phosphorylations: first by PDK1 on the activation loop (Thr308 in Akt1) followed by phosphorylation on the hydrophobic motif (Ser473 in Akt1) (Brazil and Hemmings, 2001). Phosphorylation of this second site is regulated by the TORC-2 protein complex (SIN1-mLST8-rictor-mTOR) (Jacinto et al., 2006; Sarbassov et al., 2005), although whether via direct phosphorylation or indirectly, for example by modulating the stability of the phosphate at this site, remains to be established.

Following phosphorylation on Thr308 and Ser473, Akt is locked in an active conformation and phosphorylates substrates to promote cell growth, proliferation, and cell survival. Cell-cycle effects are controlled through mechanisms that modulate the localization of cell-cycle regulators such as p27 and the E3 ubiquitin ligase HDM2 (human homolog to murine double minute 2). Akt directly phosphorylates p27, resulting in cytosolic sequestration of p27, promoting progression through the cell cycle (Viglietto et al., 2002; Zhou et al., 2001). Phosphorylation of HDM2 by Akt causes the ligase to translocate from the cytosol to the nucleus where it binds and inhibits the function of p53, subsequently promoting its translocation to the cytosol and subsequent ubiquitin-mediated degradation (Mayo and Donner, 2001). Cell-growth effects are mediated in part by TSC2 (tuberin), a tumor suppressor that forms a heterodimeric complex with TSC1 (hamartin) and is a GTPase-activating protein (GAP) for the small G protein Rheb. When GTP is bound to Rheb it activates the TORC-1 complex (mTOR-raptor). Multisite phosphorylation of TSC2 by Akt inhibits the GAP activity of TSC2 toward Rheb and thereby activates the TORC-1 complex, resulting in increased activity of p70S6K and increased cell growth. (Kwiatkowski and Manning, 2005; Marygold and Leevers, 2002). Akt mediates cell survival through phosphorylation of substrates such as GSK-3β (Jope and Johnson, 2004; Pap and Cooper, 1998; Pap and Cooper, 2002) and members of the Forkhead Box O (FoxO) family of transcription factors (Biggs et al., 1999; Datta et al., 1997). Increasing evidence supports distinct functions

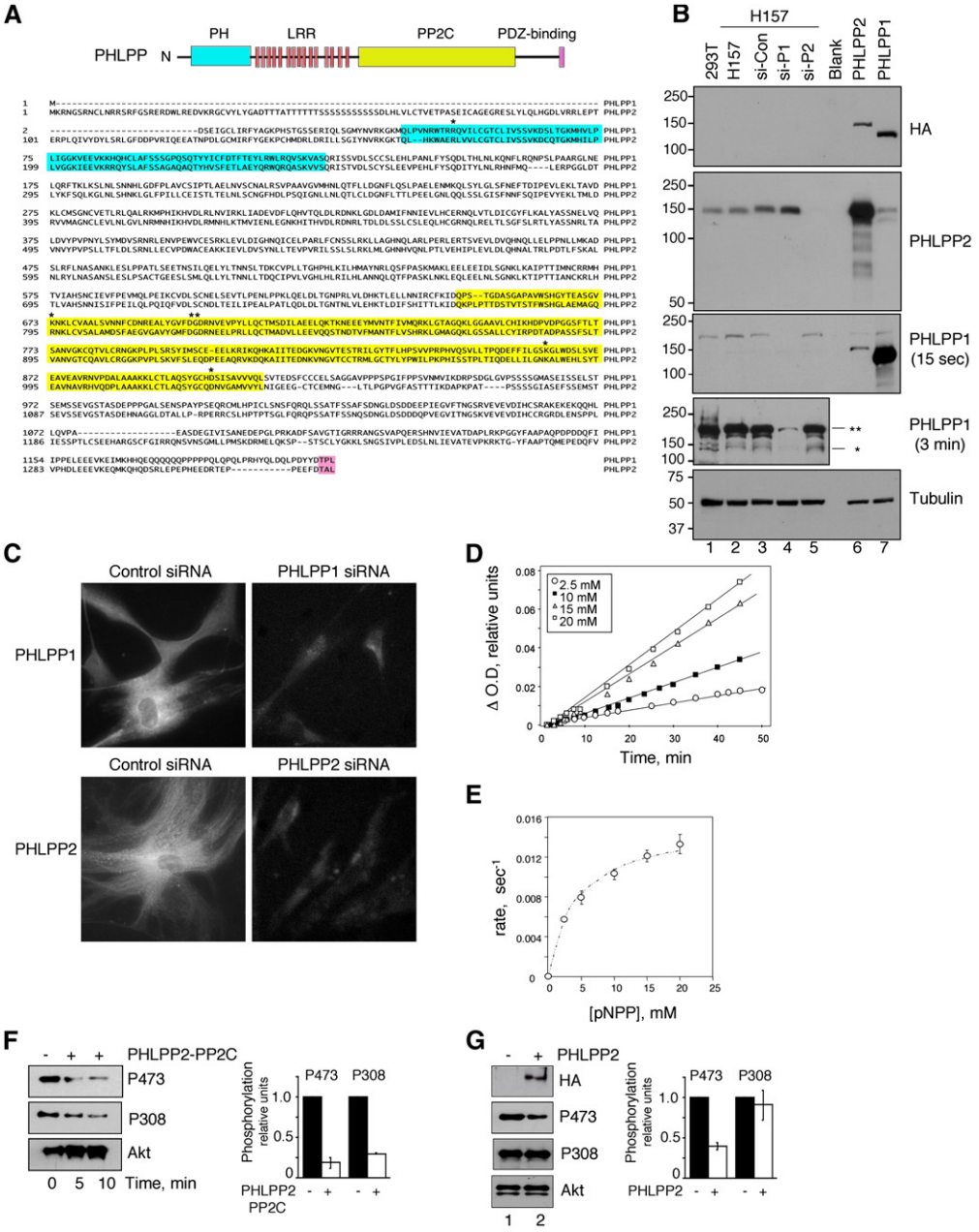

Figure 1. Domain Composition and Characterization of PHLPP2

(A) Domain structure and sequence alignment of PHLPP1 and PHLPP2 showing PH domain (cyan), leucine-rich repeat (red), phosphatase domain (yellow), and PDZ-binding motif (pink). Asterisks indicate conserved key residues within PH and PP2C domains described in Gao et al. (2005).

(B) Western blot of lysates from 293T (lane 1) or H157 NSCLC (lane 2) cells probed with isoform-specific antibodies for PHLPP1 or PHLPP2; two exposures shown for PHLPP1 blot. Asterisk denotes PHLPP1α and double asterisk denotes PHLPP1β. For migration controls, H157 lysates transfected with HA-PHLPP2 (lane 6) or HA-PHLPP1 (lane 7) were analyzed with HA antibody. H157 cells were treated with nontargeting siRNA controls (si-Con; lane 3), SMARTpool siRNA for PHLPP1 (si-P1 lane 4), or SMARTpool siRNA for PHLPP2 (si-P2; lane 5).

(C) Hs578Bst cells treated with control siRNA or isoform-specific PHLPP siRNA were stained using PHLPP1-specific (upper panel) or PHLPP2-specific (lower panel) antibodies and analyzed by immunofluorescence.

(D) Bacterially expressed GST-PP2C domain of PHLPP2 (100 nM) was incubated with the indicated concentrations of *p*-nitrophenylphosphate (*p*NPP) and the dephosphorylation continuously monitored by detecting the change in absorbance at 405 nm, as described in the Experimental Procedures. Dephosphorylation was linear with time under the conditions of this assay up to 10 hr.

(E) Initial rates of *p*NPP dephosphorylation were plotted as a function of substrate concentration. Data represent the mean ± SEM of at least three independent experiments.

for each Akt isoform. For example, studies with knockout mice have implicated Akt2 in glucose homeostasis and Akt1 in growth regulation (Cho et al., 2001a, 2001b). Furthermore, recent reports reveal that Akt1 activation suppresses cell migration and invasion whereas Akt2 promotes invasion, particularly relevant in metastasis (Irie et al., 2005; Yoeli-Lerner et al., 2005). Thus, propagation of signaling by Akt involves complex networks with multiple endpoints.

Akt signaling is terminated by two mechanisms: removal of the activating lipid second messenger, catalyzed by the lipid phosphatase PTEN (Maehama and Dixon, 1998), and dephosphorylation of activated Akt. Direct dephosphorylation of Akt is mediated by PP2A-type phosphatases (Andjelkovic et al., 1996) and a novel phosphatase our lab recently discovered named PHLPP (Gao et al., 2005). PHLPP directly dephosphorylates the hydrophobic motif of Akt (Ser473 on Akt1), resulting in inhibition of kinase activity and promotion of apoptosis (Gao et al., 2005). Failure to terminate signaling via the PI3K/Akt pathway results in increased cell growth, proliferation, and inhibition of apoptosis. Loss of acute regulation of these cellular processes is a hallmark of tumorigenesis, and many proteins in this pathway, including PI3K and PTEN, are somatically mutated in cancer (Li et al., 1997; Samuels et al., 2004).

Here we identify and characterize a second isoform of the PHLPP family, PHLPP2, and show that the PHLPP isoforms differentially terminate Akt signaling. Knockdown studies reveal that termination of defined Akt-signaling pathways is mediated through the specific interactions of PHLPP1 and PHLPP2 with Akt1, 2, or 3. Thus, signaling complexes of specific PHLPP isoforms, Akt isoforms, and Akt downstream substrates drives specificity in propagating and terminating signaling in the PI3K/Akt pathway.

RESULTS

A search of the NCBI database for novel isoforms of the phosphatase PHLPP revealed a gene predicted to encode a 1323 residue protein that we name PHLPP2. The gene is located at chromosome 16q22.3 and is comprised of 18 exons. This gene is the only other gene predicted to encode a protein with the same domain composition as the originally described PHLPP: a PH domain, leucine-rich repeats, a PP2C phosphatase domain, and a PDZ-binding motif (Figure 1A). This new isoform shares 50% overall amino acid identity with the original PHLPP, which we hereafter refer to as PHLPP1. Identity in the PH domains and PP2C domains of PHLPP1 and PHLPP2 are 63% and 58%, respectively. Key residues identified for phosphate and metal binding in PP2Cα are conserved in both PHLPP isoforms (asterisks in PP2C domain [yellow], Figure 1A) (Jackson et al., 2003). It is also noteworthy that, similar to PHLPP1, only the second Arg of the signature motif RXRXF of phosphoinositide-binding PH domain is present in PHLPP2 (asterisk in PH domain [cyan], Figure 1A) (Ferguson et al., 2000). The most striking differences are an amino-terminal extension of ~14 kDa present in PHLPP2 and divergence in C-terminal sequences, including the PDZ-binding motifs.

Human PHLPP2 was cloned as described in the Experimental Procedures and HA-tagged PHLPP2 was expressed in H157 cells (Figure 1B, lane 6). Transiently expressed HA-tagged PHLPP2 migrated with a molecular mass of ~150 kDa, consistent with the additional 129 amino acids compared to HA-PHLPP1, which migrates with an apparent molecular mass of 140 kDa (Figure 1B, lane 7). To probe for expression of endogenous PHLPP1 and PHLPP2, we used isoform-specific antibodies (Figure 1B, lanes 1–5). PHLPP2-specific antibodies detected a band comigrating with expressed PHLPP2 in 293T and H157 cells (Figure 1B, lanes 1 and 2) that was absent following knockdown of PHLPP2 (lane 5), but not PHLPP1 (lane 4), by isoform-specific siRNA. PHLPP1 was originally described as a 140 kDa protein; however, the PHLPP1-specific antibody detected a major band with an apparent molecular mass of 190 kDa (Figure 1B, PHLPP1 panel, long exposure, double asterisk) and a minor band at 140 kDa (visible in long exposure, PHLPP1 panel, asterisk) consistent with the originally described PHLPP protein. Both bands were effectively depleted in cells treated with siRNA for PHLPP1, but not PHLPP2, suggesting the upper band is a splice variant of PHLPP1. In support of this, the most recent update of gene annotation available through the NCBI database predicts a longer PHLPP1 gene (accession number O60346) containing extra 5′ sequence upstream of the original PHLPP1 start codon (Gao et al., 2005) and thus encoding a larger protein (1717 residues). Consistent with two splice variants of PHLPP1, northern blot analysis revealed expression of two transcripts of ~5 kb and 7 kb (data not shown). Thus, the PHLPP family comprises two gene products, PHLPP1 and PHLPP2, with PHLPP1 having two splice variants, which we name PHLPP1α (the originally described PHLPP [Gao et al., 2005]) and PHLPP1β. Immunohistochemistry revealed that PHLPP1 and PHLPP2 are distributed throughout the cell in the Hs578Bst normal breast cell line (Figure 1C) and H157 cell line (data not shown) with fractionation studies supporting cytosolic, membrane, and nuclear distribution for both isoforms (data not shown).

(F) Dephosphorylation of pure His-tagged Akt1 was detected following incubation with purified PHLPP2-PP2C domain for 5 (lane 2) or 10 (lane 3) min; PHLPP2-PP2C domain was omitted in lane 1. Quantification of three independent experiments showing relative phosphorylation of Akt at P308 and P473 at the 5 min time point; error bars indicate standard deviation.

(G) 293T cells were transfected with vector (lane 1) or HA-PHLPP2 (lane 2) under high serum conditions (10% FBS DMEM); thereafter HA-PHLPP2 was immunoprecipitated and incubated with pure phosphorylated Akt1 for 10 min. Akt phosphorylation was detected using phospho-specific antibodies. Bar graph summarizes data from three independent experiments; error bars indicate standard deviation.

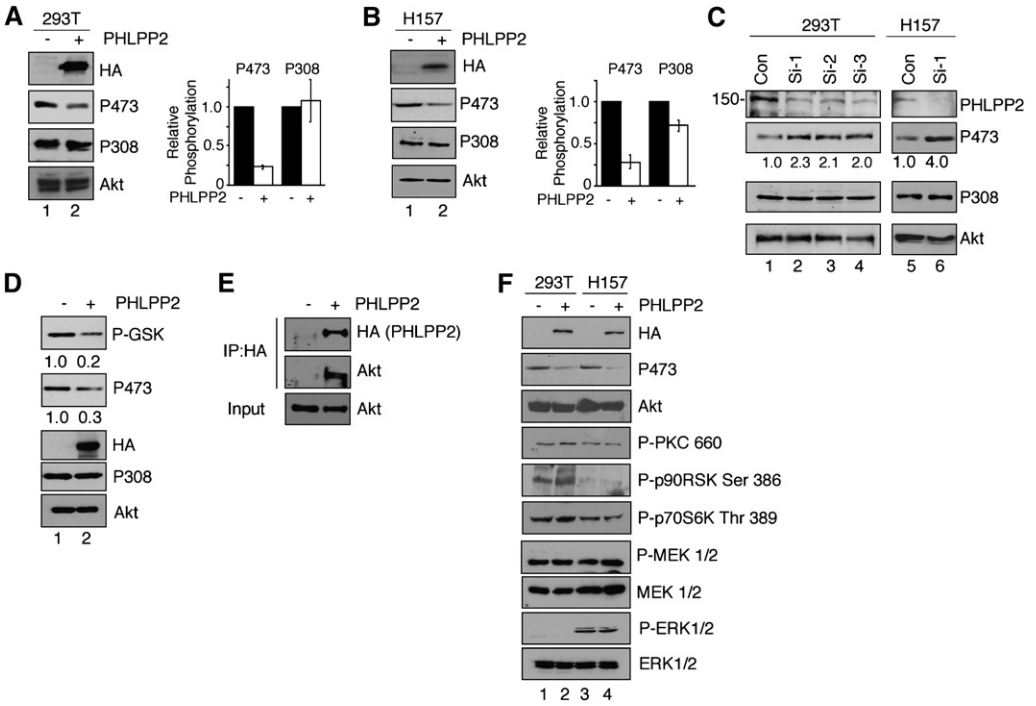

Figure 2. In Vivo Characterization of PHLPP2

(A and B) 293T and H157 cells were transfected with vector (lane 1) or HA-PHLPP2 (lane 2) for 48 hr under high serum conditions prior to lysis. The phosphorylation of Akt in lysates was detected by western blot analysis. Data from three independent experiments are summarized in bar graph (relative phosphorylation of Akt at P473 or P308 was normalized to total Akt). Error bars indicate standard deviation.

(C) Cell lysates from 293T or H157 cells transfected with control (Con) or PHLPP2-specific siRNA (Si-1, Si-2, and Si-3) for 48 hr under high serum conditions. The phosphorylation of Akt and relative protein levels of Akt and PHLPP2 were detected by western blot analysis using PHLPP2, phospho-specific Akt, and total Akt antibodies. Relative S473 phosphorylation, normalized to total Akt, is indicated below the blot. Blots are representative of three independent experiments.

(D) Akt was immunoprecipitated from 293T cells transfected with vector (lane 1) or HA-PHLPP2 (lane 2) for 48 hr under high serum conditions and incubated with a GSK-3-fusion protein in an in vitro kinase assay. GSK-3 phosphorylation was assessed by western blot analysis with phospho-specific GSK-3 antibodies. The relative phosphorylation was normalized to total Akt and quantified below the blots.

(E) HA-PHLPP2 was immunoprecipitated from 293T cells transfected with vector (lane 1) or HA-PHLPP2 (lane 2). Immunoprecipitates were subsequently analyzed by western blot analysis for the presence of Akt or PHLPP2.

(F) 293T and H157 cells were transfected with vector (lane 1) or HA-PHLPP2 (lane 2) for 48 hr under high serum conditions. The phosphorylation of Akt, ERK 1/2, MEK 1/2, PKC, p70S6K, and p90RSK in lysates was detected by western blot analysis; blot is representative of three independent experiments.

We first tested whether the PP2C domain of PHLPP2 is catalytically competent. Figure 1D shows that the PHLPP2 PP2C domain catalyzed the dephosphorylation of the synthetic phosphatase substrate *para*-nitrophenylphosphate (*p*NPP) in a concentration- and time-dependent manner. Kinetic analysis revealed a K_m of 4.13 ± 0.05 mM and k_{cat} of 0.015 ± 0.001 s^{-1} (Figure 1E). This K_m is similar to that reported for PP2Cα toward *p*NPP, but the catalytic rate toward this synthetic substrate is ~100-fold lower than that of PP2Cα. We next asked whether the PP2C domain of PHLPP2, like that of PHLPP1, dephosphorylates Akt. Incubation of purified bacterially expressed PHLPP2 PP2C domain with pure, phosphorylated Akt resulted in dephosphorylation of Ser473 and Thr308 as assessed with phospho-specific antibodies (Figure 1F; 82% ± 6% phospho-Ser473 [P473] and 70% ± 2% of phospho-Thr308 [P308] dephosphorylated in 5 min under the conditions of the assay). Thus, the isolated PP2C domain of PHLPP2 encodes a functional phosphatase domain capable of dephosphorylating synthetic substrates as well as Akt in vitro.

We next examined the phosphatase activity of full-length PHLPP2. In contrast to the isolated PP2C domain, immunoprecipitated full-length PHLPP2 specifically dephosphorylated Ser473 and not Thr308: incubation of immunoprecipitated HA-PHLPP2 with pure phosphorylated Akt resulted in 60% ± 4% dephosphorylation of Ser473 under the conditions of the assay with no significant effect on Thr308 phosphorylation (Figure 1G). These data reveal that inhibitory constraints imposed by the regulatory regions of PHLPP2 constrain its phosphatase activity so that it is specific for the hydrophobic motif of Akt.

PHLPP2 was also an effective Ser473 phosphatase in cells: overexpression of HA-PHLPP2 in 293T (Figure 2A)

and H157 cells (Figure 2B) resulted in a 76% ± 3% and 72% ± 8% reduction in phosphorylation at Ser473, respectively, with minimal effects on the phosphorylation of Thr308. Given transfection efficiencies of 70%–90% for 293T and H157 cells, these data are consistent with the overexpressed PHLPP2 catalyzing the quantitative dephosphorylation of Akt in transfected cells, resulting in significantly decreased activity. Thus, PHLPP2 directly and selectively dephosphorylates the hydrophobic motif of Akt. (Note we chose to use the H157 cells [a non-small-cell lung cancer cell line, NSCLC] because under conditions of serum deprivation, Akt inhibition has been shown to induce apoptosis [Brognard et al., 2001], thus providing a useful cell system to examine the effects of PHLPP2 on Akt-mediated apoptosis.)

To test whether endogenous PHLPP2 regulates the phosphorylation of Akt in cells, we knocked down endogenous PHLPP2 by siRNA. We generated three unique siRNAs and all resulted in greater than a 3.5-fold reduction in PHLPP2 protein in the 293T cells (Figure 2C, lanes 2–4); smartpool siRNA (combining all three siRNAs) was used for all subsequent experiments. Knockdown of PHLPP2 protein resulted in a 2-fold increase in Akt phosphorylation at Ser473 but did not significantly alter phosphorylation at Thr308 in 293T cells (Figure 2C lanes 2–4). Knockdown of PHLPP2 resulted in a 4-fold increase in Ser473 phosphorylation and no change in Thr308 phosphorylation in H157 cells (Figure 2C, lane 6). Similar results were obtained following knockdown of PHLPP2 in SKBR-3 and MCF-7 cells (Figure S1 in the Supplemental Data available with this article online).

Maximal Akt activity requires phosphorylation on both Ser473 and Thr308, leading us to address the effect of dephosphorylation by PHLPP2 on cellular Akt activity. Akt immunoprecipitated from 293T cells overexpressing PHLPP2 had markedly reduced levels of Ser473 phosphorylation compared to Akt from vector-transfected cells (Figure 2D; 70% reduction in Ser473 phosphorylation); this reduced phosphorylation correlated with reduced activity toward phosphorylation of a GSK-3 fusion protein substrate in an in vitro kinase assay (Figure 2D; 80% reduction in substrate phosphorylation). Thus, the selective dephosphorylation of Ser473 on Akt by PHLPP2 results in a dramatic decrease in kinase activity.

To determine if Akt and PHLPP2 associate in cells, we immunoprecipitated HA-PHLPP2 from 293T cells and probed for association with endogenous Akt. Figure 2E shows that endogenous Akt was present in immune complexes of HA-tagged PHLPP2, revealing that the two proteins associate in cells.

We next addressed the specificity of PHLPP2 for the hydrophobic phosphorylation motif (Ser473) of Akt relative to that of other AGC kinase family members: 70 kDa ribosomal S6 kinase (p70S6K), 90 kDa ribosomal S6 kinase (p90RSK), and protein kinase C (PKC). Expression of PHLPP2 caused a marked decrease in the phosphorylation of Akt on Ser473 but, in the same cells, had no significant effect on the phosphorylation of the hydrophobic motif of PKC, p90RSK, or p70S6K (Figure 2F). These data are consistent with PHLPP2 specifically dephosphorylating the hydrophobic motif of Akt under the conditions of our experiments. It was previously reported that a protein corresponding to PHLPP1 negatively regulates the MAPK-signaling pathway (Shimizu et al., 2003). To determine whether PHLPP2 also negatively regulates the MAPK-signaling pathway, we examined activation of this pathway in untreated cells expressing empty vector or HA-PHLPP2. PHLPP2 overexpression did not alter the phosphorylation of MEK 1/2 or ERK 1/2 under the conditions of the experiment (Figure 2F), suggesting PHLPP2 does not regulate this pathway.

The foregoing results reveal that PHLPP isoforms suppress the phosphorylation of Akt under basal conditions by selectively dephosphorylating Ser473. We next asked whether the PHLPP isoforms control the amplitude or duration of agonist-stimulated Akt phosphorylation. Treatment of Hs578Bst cells, a normal breast cell line, with EGF resulted in a rapid and transient rise in the phosphorylation state of Akt on Ser473 and Thr308. Figure 3A shows that the phosphorylation at both sites increased significantly following 15 min of EGF treatment (lane 2) but returned to baseline following 30 min treatment (lane 3). Depletion of both PHLPP1 and PHLPP2 resulted in a remarkable 30-fold increase in the EGF-stimulated phosphorylation of Akt on Ser473 and, unexpectedly, Thr308 (Figure 3A, lane 8). (Because siRNA depletes both PHLPP1α and PHLPP1β, we use PHLPP1 to denote both PHLPP1α and PHLPP1β.) This increase was sustained longer relative to control cells, with decay to PHLPP-depleted baseline levels requiring at least 24 hr for Ser473 (lane 12). Curiously, PHLPP isoforms selectively control the phosphorylation state of Ser473 under basal conditions (10% FBS DMEM) but control the phosphorylation state of Ser473 and Thr308 following acute agonist stimulation. These data establish that PHLPP isoforms play a major role in controlling the amplitude of agonist-dependent phosphorylation of Akt. Qualitatively similar results were observed in H157 cells (data not shown).

We next compared the effects of depletion of PHLPP1 or PHLPP2 individually on the agonist-dependent phosphorylation of Akt. Consistent with the results in Figure 2C for 293T and H157 cells, depletion of either PHLPP1 (lane 2), PHLPP2 (lane 3), or both (lane 4) in Hs578Bst cells caused an increase in the basal phosphorylation state of Ser473, but not Thr308 (Figure 3B). Stimulation with EGF for 15 min caused an increase in the phosphorylation of Ser473 and Thr308 (lane 5) that was highly dependent on PHLPP1 and PHLPP2: depletion of either isoform caused a striking increase in the phosphorylation of Ser473 and Thr308. Knockdown of both isoforms resulted in a comparable increase to that observed following single knockdown. Thus, both PHLPP1 and PHLPP2 set the amplitude and duration of the Akt signal.

The finding that dephosphorylation by PHLPP2 inactivates Akt led us to hypothesize that the cellular processes controlled by Akt are regulated by PHLPP2. To address

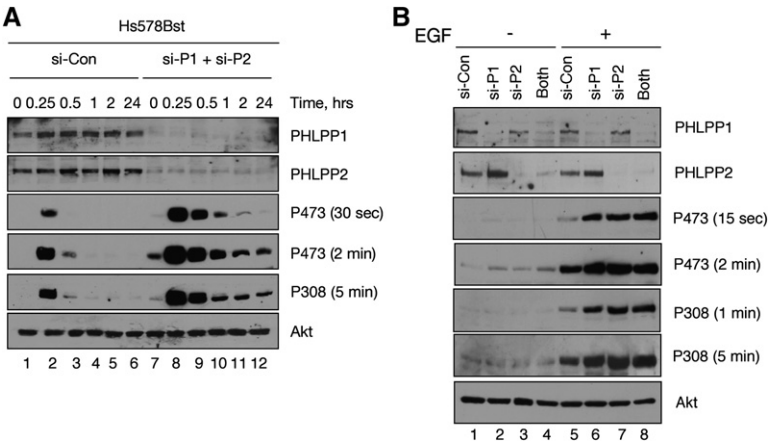

Figure 3. PHLPP Isoforms Control the Amplitude of Agonist-Stimulated Akt Phosphorylation

(A) Hs578Bst cells, a normal breast cell line, were transfected with SMARTpool siRNA to both PHLPP1 and PHLPP2 under high serum conditions and incubated for 48 hr. Media were then changed to low serum (0.1% FBS DMEM) and cells incubated 2 hr prior to addition of EGF (10 ng/ml) for 24 hr time point or incubated overnight in low serum media for all other EGF time points. The phosphorylation of Akt and protein levels of Akt, PHLPP1, and PHLPP2 in lysates were detected by western blot analysis; exposure times indicated for phosphoblots.

(B) Hs578Bst cells were transfected with SMARTpool siRNA to PHLPP1, PHLPP2, or both under high serum conditions and incubated for 48 hr. Media were then changed to low serum and cells cultured overnight prior to addition of EGF (10 ng/ml) for 15 min. The phosphorylation of Akt and protein levels of Akt, PHLPP1, and PHLPP2 in lysates were detected by western blot analysis.

this possibility, we examined the effects of expressing PHLPP2 on Akt-mediated apoptosis. Expression of PHLPP2 resulted in an increase in apoptosis in H157 cells under conditions of serum deprivation: The relative sub-2N DNA increased an order of magnitude from 3.1% ± 0.1% in vector-transfected cells to 28% ± 5% in PHLPP2-transfected cells (Figure 4A). To examine if PHLPP2 regulated Akt-mediated apoptosis in other cancer cell lines, we expressed HA-PHLPP2 in the Bt-474 and MDA-MB-231 breast cancer cell lines: expression of PHLPP2 resulted in an ~80% and 70% decrease in phosphorylation at Ser473 (Figure 4B). Comparable results were observed in the ZR-75-1 and MCF-7 breast cancer cell lines (data not shown). Furthermore, expression of PHLPP2 in breast cancer cells resulted in an increase in apoptosis (from 2.0 ± 0.2 to 15.8 ± 0.6 relative units in Bt-474 cells and from 2.9 ± 0.9 to 12 ± 4 relative units in MDA-MB-231 cells), as assessed by quantifying sub-2N-DNA content (Figure 4B). These data reveal that, as noted previously for PHLPP1, PHLPP2 also promotes apoptosis.

To more rigorously explore the role of the PHLPP isoforms in mediating apoptosis, we examined the effect of depletion of PHLPP1, PHLPP2, or both isoforms on apoptosis triggered by the DNA-damaging agent Etoposide. Treatment of H157 cells with Etoposide resulted in a 5-fold increase in apoptosis (Figure 4C). Depletion of PHLPP1, PHLPP2, or both isoforms caused a 2-fold reduction in Etoposide-mediated apoptosis. These results reveal that PHLPP isoforms promote apoptosis both under basal conditions and following exposure to cytotoxic agents.

We next tested whether the increased apoptosis observed in cells overexpressing PHLPP2 resulted from PHLPP2-mediated dephosphorylation of Ser473 on Akt. To this end, we coexpressed PHLPP2 with a phosphomimetic, and thus constitutively active, Akt construct (S473D) in MDA-MB-231 cells. Importantly, we found that the PHLPP2-resistant Akt rescued two-thirds of the apoptosis induced by PHLPP2 (Figure 4D). Thus, PHLPP2 negatively regulates Akt, resulting in the induction of apoptosis, and this effect can be rescued by a phosphomimetic Akt construct resistant to dephosphorylation by PHLPP2.

Akt has been reported to regulate both proliferation and cell-cycle entry, leading us to ask whether PHLPP2 affects cell-cycle progression. Expression of PHLPP2 resulted in a ~3-fold and 2-fold increase in the G1/S ratio (as assessed by flow cytometry) in both 293T and H157 cells, respectively (Figure 4E), suggesting cells were entering the cell cycle at a decreased rate. Consistent with this result, knockdown of endogenous PHLPP2 in H157 cells caused a 2-fold decrease in the G1/S ratio indicating that the cells in which PHLPP2 was depleted were proliferating at an increased rate (Figure 4F). Interestingly, despite knockdown of either PHLPP1 or PHLPP2 causing a comparable increase in Ser473 phosphorylation (see Figures 4G and 5A–5C), PHLPP1 depletion resulted in a smaller decrease (~25%) in the G1/S ratio compared to the ~50% decrease resulting from PHLPP2 depletion. Consistent with decreased levels of PHLPP2 causing a selective increase in cell proliferation in the H157 cells, BrdU incorporation increased 1.7-fold in cells in which PHLPP2 was knocked down and only 1.3-fold in cells in which PHLPP1 was knocked down (Figure 4G). The combined knockdown of both PHLPP1 and PHLPP2 did not cause BrdU incorporation to differ significantly from the increase resulting from PHLPP2 knockdown alone. Importantly, similar increases in BrdU incorporation were observed in the normal breast cell line Hs578Bst (Figure 4G). Western blot analysis of the cells used for the BrdU analysis verified that PHLPP1 and PHLPP2 had been selectively knocked down and revealed that Ser473 phosphorylation increased for both knockdowns (Figure 4G). These data reveal that both PHLPP1 and PHLPP2 control cell proliferation by regulating the activation state of Akt, with PHLPP2 having a more pronounced effect.

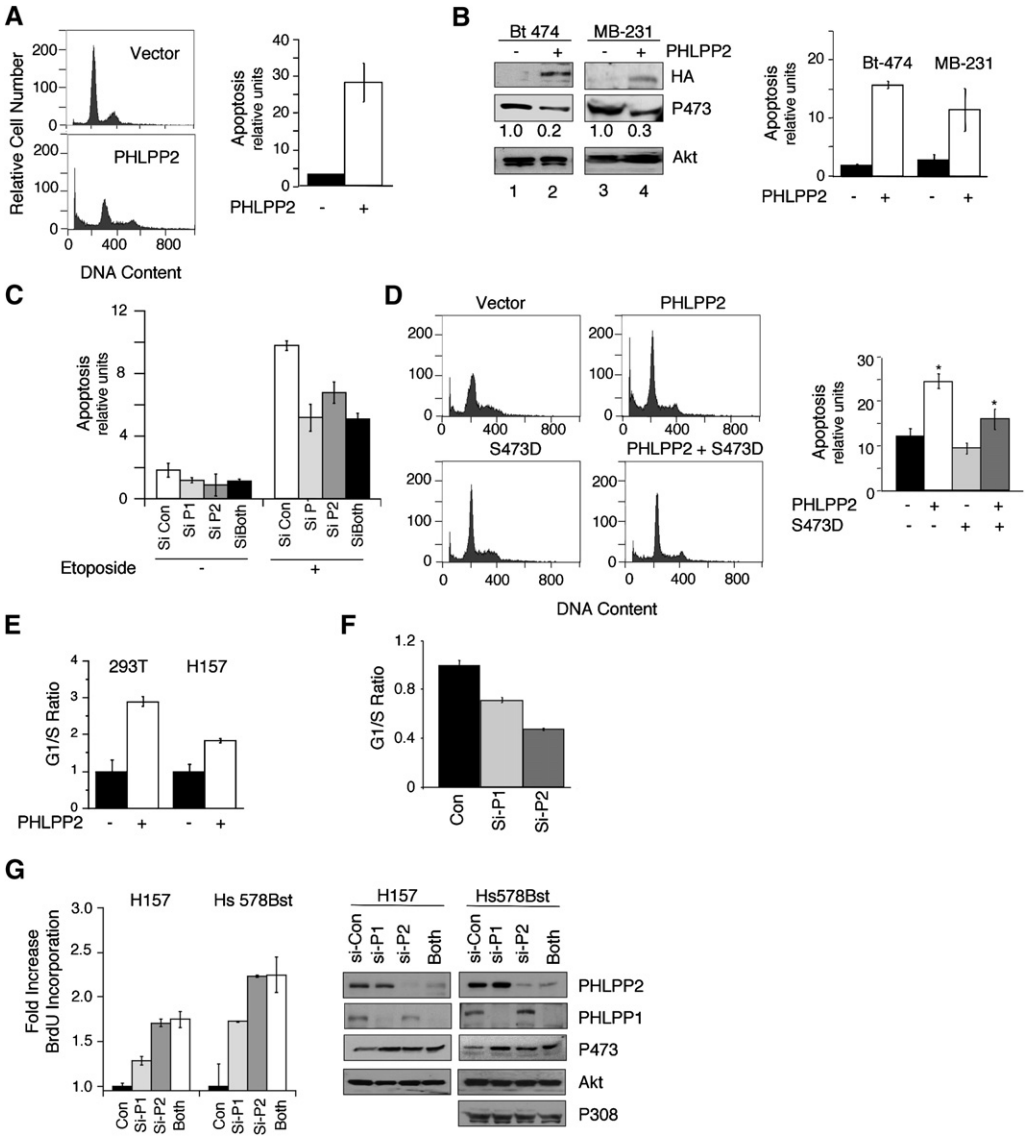

Figure 4. PHLPP2 Regulates Apoptosis and the Cell Cycle

(A) H157 NSCLC cells were transfected with HA-PHLPP2 or vector under low serum conditions (0.1% FBS DMEM) for 48 hr, and apoptosis (sub-2N DNA content) was assessed using propidium iodide incorporation assays and flow cytometry.

(B) HA-PHLPP2 or vector alone was expressed in breast cancer cell lines for 48 hr under low serum conditions and the phosphorylation of Akt in lysates was detected by western blot analysis. Relative phosphorylation is normalized to total Akt. Apoptosis was assessed using propidium iodide incorporation assays and flow cytometry in cells expressing HA-PHLPP2 or vector alone.

(C) H157 cells were transfected with SMARTpool siRNA to PHLPP1, PHLPP2, or both under high serum conditions and incubated for 48 hr. Media was then changed to low serum conditions, etoposide (50 μM) was added for 24 hr, and apoptosis was measured by flow cytometry.

(D) Expression of Akt S473D rescues PHLPP2-induced apoptosis. MDA-MB-231 cells were transfected with indicated constructs for 48 hr under low serum conditions. Histograms show sub-2N DNA; quantitation of sub-2N DNA is indicated in bar graph. One-way ANOVA with posthoc Student's t test was performed on data from S473D and PHLPP2 transfections and compared to empty vector control; asterisks indicate $p < 0.01$.

(E) An increase was observed in the G1/S ratio in cells transfected with HA-PHLPP2 compared to cells transfected with vector alone. Cells were transfected for 48 hr under high serum conditions, and the G1/S ratios were determined by propidium iodide incorporation assays and flow cytometry.

(F) Knockdown of PHLPP1 (Si-P1) or PHLPP2 (Si-P2) for 48 hr under high serum conditions decreased G1/S ratio in H157 cells as assessed by flow cytometry.

(G) Knockdown of PHLPP1, PHLPP2, or both for 48 hr under high serum conditions increased BrdU incorporation in H157 and Hs578Bst cells. Western blots were performed in parallel to ensure the PHLPP phosphatases were being sufficiently knocked down.

For all panels, data in bar graphs are representative of assays performed in triplicate, with error bars indicating standard deviation, and are representative of three independent experiments.

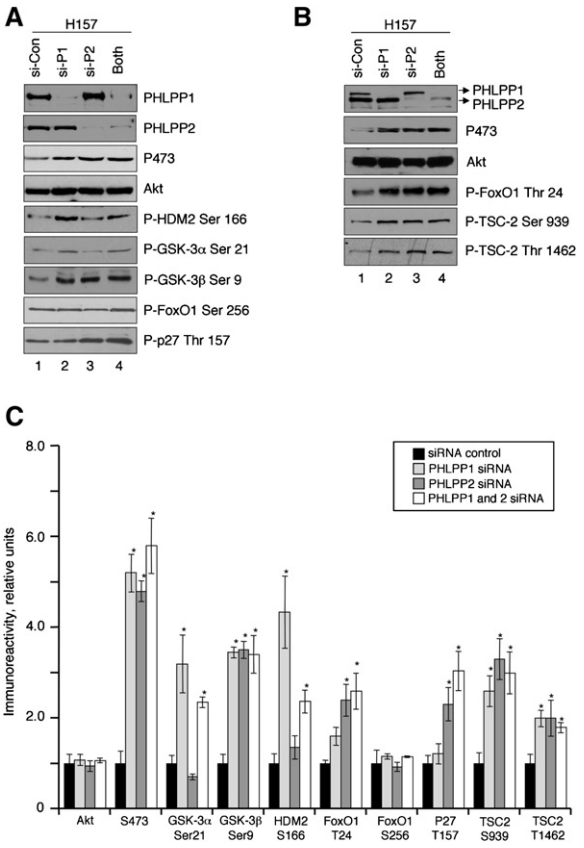

Figure 5. PHLPP1 and PHLPP2 Differentially Regulate Akt Downstream Substrates

(A) H157 cells were transfected with nontargeting siRNA control (si-Con), PHLPP1 SMARTpool siRNA (si-P1), PHLPP2 SMARTpool siRNA (si-P2), or SMARTpool siRNA for both PHLPP1 and PHLPP2 (both) for 48 hr under high serum conditions, and lysates were analyzed by western blot. The phosphorylation state of Akt, HDM2, GSK-3α/β, FoxO1, and p27 and relative protein levels of Akt, PHLPP1, and PHLPP2 were detected using the indicated phospho-specific and total endogenous protein antibodies. Western blots are representative of three independent experiments.

(B) As in (A), H157 cells were transfected with SMARTpool siRNA to PHLPP1, PHLPP2, or both, incubated 48 hr under high serum conditions, and lysates were analyzed by western blot. The phosphorylation state of FoxO1, Akt, and TSC2 and relative protein levels of Akt, PHLPP1, and PHLPP2 were detected using indicated antibodies. Western blots are representative of three independent experiments.

(C) Quantification of phosphorylation of immunoreactivity of three independent experiments as in (A) and (B). One-way ANOVA with post-hoc Student's t test was performed on all data from siRNA treatments compared to control; asterisks indicate $p < 0.05$. Error bars indicate standard error of the mean from three independent experiments.

To address the mechanism driving the more pronounced effects on the cell cycle by PHLPP2 compared with PHLPP1, we examined the effect of knocking down each PHLPP isoform alone or in combination on the phosphorylation state of downstream substrates of Akt. Figure 5A shows that PHLPP1 (lane 2) and PHLPP2 (lane 3) were effectively and specifically knocked down with isoform-specific siRNA treatment in H157 cells, resulting in a 5-fold increase in the phosphorylation of Ser473 (lanes 2 and 3, P473 panel; data from three independent experiments quantified in Figure 5C). Knockdown of both isoforms did not further increase Ser473 phosphorylation relative to selective knockdown of each isoform individually (lane 4, P473 panel). Knockdown of PHLPP1, but not PHLPP2, specifically increased the phosphorylation of two Akt substrates: HDM2 (P-HDM2 panel; phosphorylation of Ser166) and GSK-3α (P-GSK-3α; phosphorylation of Ser21). Knockdown of PHLPP2, but not PHLPP1, specifically increased the phosphorylation of p27 on Thr157 (P-p27 panel). Knockdown of either PHLPP1 or PHLPP2 increased the phosphorylation of GSK-3β (P-GSK-3β; phosphorylation of Ser9) and TSC2 (P-TSC2; phosphorylation on Ser 939 and Thr 1462) (Figures 5A and 5B, respectively). Interestingly, knockdown of either PHLPP1 or PHLPP2, or both isoforms, did not significantly alter the phosphorylation of FoxO1 on Ser256 (Figure 5A; P-FoxO1). However, knockdown of either isoform caused an increase in the phosphorylation of Thr24 on FoxO1 (Figure 5B, P-FoxO1 Thr24 panel) that was more significant for the PHLPP2 knockdown compared to the PHLPP1 knockdown (Figure 5C). The results of three independent experiments are quantified in Figure 5C. These data reveal that although PHLPP1 and PHLPP2 both dephosphorylate the same residue on Akt, a subset of downstream targets of Akt are differentially modulated depending on which phosphatase is depleted.

We next tested the hypothesis that the PHLPP isoforms differentially regulate distinct Akt isoforms, providing a possible mechanism underlying differences in substrate regulation. In this regard, Akt isoforms have been reported to regulate unique downstream substrates (Cho et al., 2001a, 2001b; Jiang et al., 2003). To address this, we depleted cells of PHLPP1, PHLPP2, or both isoforms, immunoprecipitated each Akt isoform, and examined the phosphorylation of Ser473. Western blot analysis of Akt immunoprecipitates (Figure 6A) revealed that knockdown of PHLPP1 caused an increase in the phosphorylation of the hydrophobic motif of Akt2 (lane 6, P473 panel), but not Akt1 (lane 2, P473 panel). Conversely, knockdown of PHLPP2 caused an increase in the phosphorylation of the hydrophobic motif of Akt1 (lane 3, P473 panel) but not Akt2 (lane 7, P473 panel). Knockdown of either PHLPP isoform increased the phosphorylation of the hydrophobic motif of Akt3 (lanes 10 and 11, P473 panel). Note that under these conditions activation loop phosphorylation was not affected by knockdown of individual PHLPP isoforms (P308 panel). These data reveal that PHLPP2 controls the phosphorylation of the hydrophobic motif on Akt1 and Akt3 and PHLPP1 controls the phosphorylation of the hydrophobic motif on Akt2 and Akt3.

We also examined whether PHLPP isoforms selectively interacted with Akt isoforms in cells by immunoprecipitating endogenous Akt isoforms and probing for endogenous PHLPP isoforms. Supporting the results of effects of PHLPP isoforms on hydrophobic motif phosphorylation

of Akt isoforms, PHLPP1 immunoprecipitated with Akt2 and Akt3 (Figure 6A, PHLPP1 panel; lanes 5/7 and 9/11), whereas PHLPP2 immunoprecipitated with Akt1 and Akt3 (Figure 6A, PHLPP2 panel; lanes 1/2 and 9/10). Note that this selectivity was lost in overexpression studies: Overexpressed PHLPP1 and PHLPP2 bound all three Akt isoforms (Figure S2). Thus, the data presented in Figure 6A establish that: (1) Akt1 binds to and is specifically dephosphorylated at the hydrophobic motif by PHLPP2, (2) Akt2 binds to and is specifically dephosphorylated at this motif by PHLPP1, and (3) both PHLPP isoforms bind and regulate the dephosphorylation of Akt3.

To test whether the isoform-specific effects of PHLPP knockdown on Akt substrates resulted from differential dephosphorylation of Akt isoforms, we depleted each Akt isoform by specific siRNA and examined the phosphorylation status of downstream substrates. The western blot in Figure 6B shows that knockdown of Akt2 (lane 3), but not Akt1 (lane 2) or Akt3 (lane 4), resulted in a decrease in phosphorylation of HDM2 (Ser166) and GSK-3α (Ser21), in agreement with the increases observed following knockdown of PHLPP1 (Figure 5A). Depletion of Akt3 (lane 4), but not Akt1 or Akt2, resulted in a dramatic decrease in p27 phosphorylation (Thr157), in agreement with the robust increase in phosphorylation following knockdown of PHLPP2. Interestingly, although PHLPP1 also regulates Akt3, PHLPP1 knockdown did not affect p27 phosphorylation (Figure 5A). Perhaps unique spatial regulation within the cell controls specificity in the PHLPP2-Akt3-p27 pathway. Depletion of each Akt isoform resulted in a decrease in GSK-3β phosphorylation, consistent with PHLPP knockdown studies showing that both isoforms of the phosphatase increase the phosphorylation of this substrate (Figures 5A and 6B). The phosphorylation of Thr24 on FoxO1 was decreased following knockdown of either of the three Akt isoforms, whereas the phosphorylation of Ser256 was unaffected. These results are consistent with the PHLPP knockdown results: phosphorylation of Thr24, but not Ser256, was increased following PHLPP knockdown (Figure 5C). Lastly, knockdown of Akt1 or Akt2, but not Akt3, caused a decrease in the phosphorylation of both Ser939 and Thr1462 on TSC2, again in agreement with the effects of PHLPP knockdown. Note that depletion of Akt1 did not decrease total Ser473 phosphorylation significantly (lane 2, Ser473 panel), likely due to upregulation of Akt2 (compare lanes 1 and 2; Akt2 panel). Data from three independent experiments are quantified in Figure 6C.

As a test of the model that PHLPP isoforms differentially inactivate Akt isoforms thus differentially terminating signaling pathways, we asked whether the effects of a specific PHLPP isoform would be abolished if its partner-Akt was also depleted. We chose to test the model with the PHLPP2-Akt3-p27 signaling complex because of the specificity of this particular interaction (only PHLPP2 [not PHLPP1] and only Akt3 [not Akt1 or Akt2] controls p27 phosphorylation). Figure 6D shows that knockdown of PHLPP2 caused an increase in the phosphorylation of p27 on Thr157 (lane 3) relative to control (lane 1) or cells depleted in Akt3 (lane 2) in the Hs578Bst primary breast cell line following EGF stimulation. This increase was abolished upon knockdown of both PHLPP2 and Akt3 (lane 4). Note that knockdown of Akt3 alone did not decrease p27 phosphorylation; this experiment differed from the ones in Figure 5A in that phosphorylation was monitored after acute ligand-driven stimulation under conditions of serum deprivation, a condition that could activate Akt isoforms to compensate for the lack of Akt3. Consistent with this, P473 staining was the same in the si-Con and si-Akt3 lanes, contrasting with results from cells grown under basal conditions (10% FBS) where knockdown of Akt3 results in a noticeable reduction in the P473 signal (e.g., Figure 6B). These data reveal that the ability of PHLPP2 to modulate the phosphorylation of p27 depends on Akt3, supporting the model that specific PHLPP isoforms control the activity of specific Akt isozymes, in turn controlling the phosphorylation of specific downstream substrates.

An intriguing finding from the foregoing experiments was that simultaneous knockdown of both PHLPP isoforms did not result in an additive increase in Ser473 phosphorylation or the phosphorylation of downstream substrates of Akt (see quantification in Figure 5C: knockdown of either PHLPP1, PHLPP2, or both PHLPP isoforms caused a 5-fold increase in Ser473 phosphorylation and, for example, a 4-fold increase in GSK-3β phosphorylation). One possibility is that depletion of both PHLPP isoforms activates an inhibitory feedback signal. A candidate for such feedback regulation is p70S6K, whose activation has been established to result in decreased phosphorylation and activation of Akt (Harrington et al., 2004; O'Reilly et al., 2006; Um et al., 2004). To test this possibility, we asked whether inhibition of p70S6K activation by rapamycin (TORC-1 inhibitor) unmasked any potential additivity in the knockdown of PHLPP1 and PHLPP2. Figure 6E shows that, as presented in earlier experiments, knockdown of PHLPP1 (lane 2), PHLPP2 (lane 3), or both PHLPP isoforms (lane 4) resulted in a comparable increase in Ser473 phosphorylation. Knockdown of either PHLPP1 or PHLPP2 had no significant effect on the phosphorylation of Thr308. However, a modest decrease in Thr308 phosphorylation was observed when both PHLPP isoforms were depleted (P308 blot, lane 4). Treatment of cells with rapamycin for 24 hr (Figure 6E) or 4 hr (data not shown) resulted in decreased overall Ser473 phosphorylation. However, depletion of either PHLPP1 or PHLPP2 increased Ser473 phosphorylation in rapamycin-treated cells (lanes 6 and 7). In marked contrast to untreated cells, rapamycin treatment doubled the level of Ser473 phosphorylation in cells in which both PHLPP isoforms had been knocked down (lane 8) compared to cells in which the PHLPP isoforms had been knocked down individually (lanes 6 and 7). Additionally, the phosphorylation of Thr308 was no longer reduced in the double-knockdown cells compared to the single-knockdown cells (compare

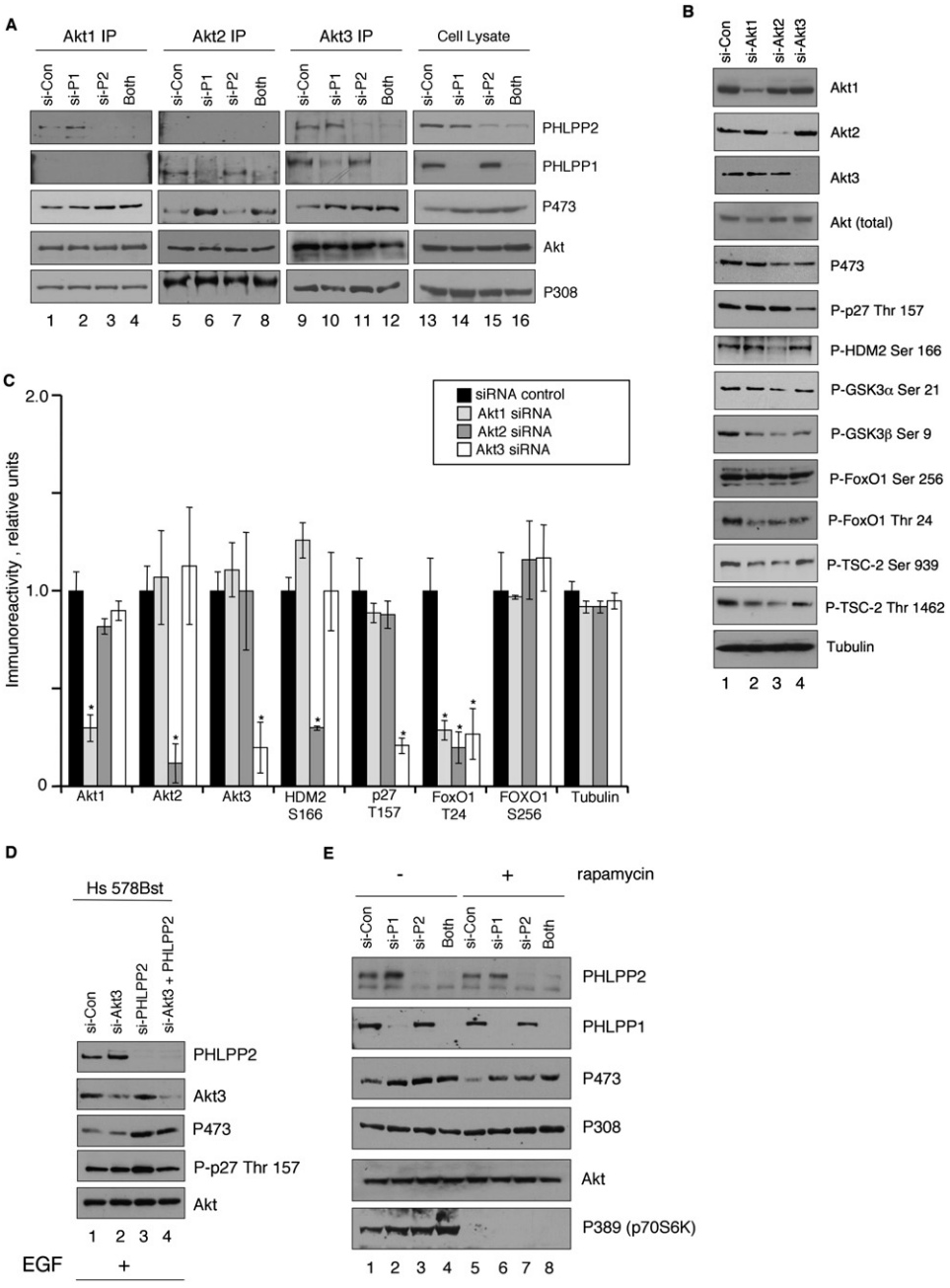

Figure 6. PHLPP1 and PHLPP2 Regulate Specific Akt Isoforms

(A) Akt isoforms were immunoprecipitated from H157 cells transfected with nontargeting control siRNA (si-Con), PHLPP1 SMARTpool siRNA (si-P1), PHLPP2 SMARTpool siRNA (si-P2), or siRNA for both PHLPP1 and PHLPP2 (both) for 48 hr under high serum conditions. Immunoprecipitates were analyzed by western blot analysis for the presence of Akt, Akt phosphorylation on Ser473 (P473), or Thr308 (P308), PHLPP1, or PHLPP2.

(B) Akt isoforms were knocked down with Akt isoform-specific siRNA for 48 hr under high serum conditions and cell lysates were analyzed by western blot with the following antibodies: Akt isoform-specific antibodies, Akt pan antibody (Akt total), Ser473 antibody (P473), and phospho-specific antibodies to indicated downstream substrates. α-tubulin was used as a loading control. Western blots are representative of three independent experiments.

(C) Quantification of immunoreactivity of indicated bands from three independent experiments as described in (B). One-way ANOVA with posthoc Student's t test was performed on data from all siRNA treatments compared to control; asterisks indicate $p < 0.05$. Error bars indicate standard error of the mean.

(D) Hs578Bst cells were transfected with siRNA to Akt3, PHLPP2 or both, incubated 48 hr under high serum conditions. Media were changed to low serum overnight and EGF (10 ng/ml) was added for 15 min prior to cell lysis. Lysates were assessed by western blot analysis for phospho-Akt, phospho-p27, Akt, Akt3, and PHLPP2.

lane 8 to lanes 6 and 7). Note that the degree of Ser473 phosphorylation (but not Thr308) was generally reduced in rapamycin-treated cells (compare lanes 1–4 to lanes 5–8). This result could be explained by newly synthesized mTOR-binding rapamycin, thus decreasing the levels of the TORC-2 complex, which would result in a decrease in Ser473 phosphorylation (Sarbassov et al., 2006). Consistent with activation of a p70S6K feedback loop when both PHLPP isoforms are depleted, knockdown of PHLPP1 or PHLPP2 alone did not dramatically increase p70S6K phosphorylation, but knockdown of both isoforms resulted in a robust increase in phosphorylation of this kinase (Figure 6E). In summary, these data reveal that depletion of both PHLPP isoforms activates a negative feedback loop mediated by p70S6K that counteracts the direct effects of PHLPP depletion on Ser473 phosphorylation.

DISCUSSION

Here we identify a second isoform of the protein phosphatase PHLPP, which we name PHLPP2. We show that both PHLPP1 and PHLPP2 selectively dephosphorylate the same site on Akt, the hydrophobic phosphorylation motif, yet the two phosphatases control different downstream substrates of Akt. We identify the mechanism for the differential signal termination as deriving from specificity in the binding and regulation of specific PHLPP isoforms with specific Akt isoforms.

PHLPP2 Dephosphorylates and Inactivates Akt

PHLPP2, like PHLPP1, selectively dephosphorylates the hydrophobic motif of Akt, resulting in decreased kinase activity, increased apoptosis, and inhibition of cell-cycle progression. Although the isolated PP2C domain is capable of dephosphorylating Thr308 and Ser473, the full-length protein has specificity for Ser473. These data reveal that the regulatory domains of PHLPP constrain substrate phosphorylation, resulting in the full-length protein discriminating between phosphorylation sites within the kinase core of Akt. The primary mechanism for the cellular effects of PHLPP2 presented are consistent with direct dephosphorylation of Ser473 of Akt because the phosphomimetic Akt S473D is able to rescue the effects of PHLPP2 overexpression (see Figure 4D). Although a number of AGC kinases share the hydrophobic phosphorylation motif, under the conditions of our experiments, we show that overexpressed PHLPP2 does not dephosphorylate the hydrophobic motif of PKC, p70S6K, or p90RSK. Nor does it regulate the MEK/ERK pathway. Thus, PHLPP2 directly and specifically regulates Akt under the conditions described.

PHLPP Isoforms Control the Amplitude of Agonist-Dependent Signaling by Akt

PHLPP isoforms directly set the amplitude of the Akt signal: depletion of either PHLPP1 or PHLPP2 causes a dramatic increase in the agonist-stimulated phosphorylation of Akt. Interestingly, although the dephosphorylation of Akt following agonist stimulation was slowed in cells lacking PHLPP, the phosphorylation of Akt returned to basal levels. These data suggest that an additional phosphatase controls the duration of agonist-evoked activation of Akt in the absence of the PHLPP phosphatases. One possibility is that a phosphatase directed at the PDK-1 site drives this deactivation, with dephosphorylation at this site destabilizing the phosphate at the hydrophobic motif. A likely candidate is a PP2A-type phosphatase, which has been shown to regulate Akt phosphorylation (Andjelkovic et al., 1996). Note that the phosphorylation of the PDK-1 site has marked sensitivity to okadaic acid; the hydrophobic site (Ser473) is only modestly affected by okadaic acid, consistent with a PP2C family member controlling this site (Gao et al., 2005). Live cell imaging studies of Akt activity have previously established that phosphatases are powerful "brakes" to Akt signaling (Kunkel et al., 2005). Here we show that PHLPP isoforms exert enormous suppression on the acute agonist-mediated phosphorylation of Akt, thus setting the amplitude of the signal.

Curiously, the phosphorylation states of both Thr308 and Ser473 were coordinately elevated following acute agonist stimulation in cells lacking PHLPP1 or PHLPP2. In contrast, only the phosphorylation state of Ser473 was affected in cells lacking PHLPP isoforms under basal conditions (10% FBS). One possibility is that the phosphorylation state of the hydrophobic motif regulates the stability of the phosphate on the activation loop. This is indeed the case with Akt's close cousin, protein kinase C: negative charge at the hydrophobic site renders the phosphate on the activation loop relatively resistant to dephosphorylation (Bornancin and Parker, 1997; Edwards and Newton, 1997). Evidence suggests this could be the case for Akt: mutation of Ser473 to Asp results in a construct of Akt that is more heavily phosphorylated on Thr308 compared to wild-type Akt (Biondi et al., 2001). Thus, it may be that the enormous increase in Ser473 phosphorylation resulting from depletion of PHLPP stabilizes the activation loop site, rendering it less sensitive to cellular phosphatases. This stabilization may not be apparent under basal conditions because the elevation in Ser473 phosphorylation is one order of magnitude lower than the agonist-stimulated elevation in Ser473 phosphorylation. Another possibility is that increased phosphorylation at Ser473 confers a more favorable conformation for PDK-1 phosphorylation; in support of this hypothesis, mutation of Ser473 to Asp increases PDK-1

(E) Rapamycin treatment unmasks additivity in PHLPP1 and PHLPP2 knockdown experiments. H157 cells were transfected with SMARTpool siRNA to PHLPP1, PHLPP2, or both, incubated for 48 hr under high serum conditions and then treated with rapamycin (100 nM) for 24 hr. Lysates were analyzed by western blotting using phospho-Akt, phospho-p70S6K, Akt, PHLPP1, and PHLPP2. Western blot is representative of three independent experiments.

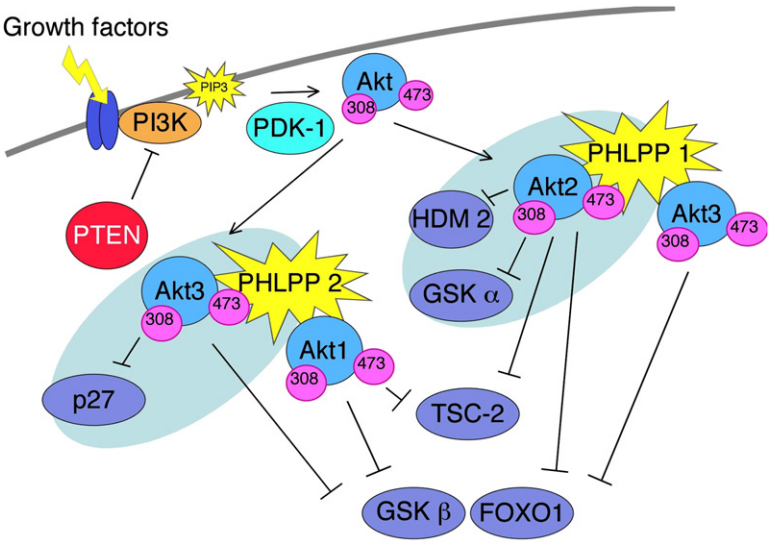

Figure 7. Model Illustrating How Specificity in Signal Termination by PHLPP Isoforms Is Achieved by Specific PHLPP-Akt-Substrate Complexes

Akt is activated following receptor-mediated activation of PI3K to generate PtdIns 3P (PIP_3). This second messenger recruits Akt to the plasma membrane, where it is phosphorylated by PDK-1 at the activation loop (Thr308), an event that triggers phosphorylation at the hydrophobic motif (Ser473). Akt then redistributes to specific intracellular locations, presumably in complex with specific substrates. Signal termination is achieved at the initial step by the lipid phosphatase PTEN, which removes the activating lipid, or, once signaling has been initiated, by the protein phosphatase PHLPP, which dephosphorylates Ser473 on Akt. Specific complexes (blue shaded ovals) of PHLPP1 or PHLPP2 with Akt1, Akt2, or Akt3 allow PHLPP isoforms to differentially terminate Akt signaling. For example, compartmentalization of PHLPP2-Akt3-p27 (blue shaded oval on left) and PHLPP1-Akt2-HDM2/GSK-3α (blue shaded oval on right) define unique pathways in the Akt signaling network. Some substrates, such as FoxO1, GSK-3β, and TSC2 are inactivated by both PHLPP isoforms via regulation of all three Akt isoforms.

phosphorylation at Thr308 in in vitro kinase assays (Biondi et al., 2001). This interplay between the two phosphorylation sites is consistent with X-ray structures of Akt, which suggest that phosphorylation of the hydrophobic motif orders the activation loop (Huang et al., 2003; Yang et al., 2002).

PHLPP Isoforms Differentially Regulate Signaling by Akt Isoforms

Akt regulates proliferation and apoptosis through multiple mechanisms. Here we define signaling networks regulated by unique Akt isoforms whose amplitude is controlled by specific PHLPP isoforms (Figure 7). Knockdown studies reveal that PHLPP2 is the dominant phosphatase in controlling the cell cycle. Furthermore, it specifically opposes the action of Akt3 on the phosphorylation state of p27: knockdown of Akt3 (but not Akt1 or 2) decreased p27 phosphorylation, whereas knockdown of PHLPP2 (but not PHLPP1) increased p27 phosphorylation. Specific signaling scaffolds are implicated by our finding that PHLPP1 binds and dephosphorylates Akt3, yet p27 phosphorylation is not sensitive to PHLPP1 depletion. Thus, a PHLPP2-Akt3-p27 pathway is suggested by our data. PHLPP1, on the other hand, regulates the phosphorylation of HDM2 and GSK3-α, and our data suggest that the mechanism is by dephosphorylation of Akt2. Specific regulation of GSK3-α by Akt2 has been previously reported (Jiang et al., 2003). We also identify a group of substrates whose phosphorylation is controlled by all three Akt isoforms and both PHLPP isoforms. For example, the phosphorylation state of GSK-3β and FoxO1 increases following knockdown of either PHLPP isoform and decreases following knockdown of any of the Akt isoforms. The overlapping regulation of these proteins by both PHLPP isoforms is consistent with the apoptotic effects induced by both phosphatases. Curiously, our data reveal that PHLPP and Akt selectively regulate specific residues within the same substrate: under conditions where the phosphorylation state of FoxO1 at Thr24 was impacted by PHLPP knockdown, no effects on the phosphorylation of Ser256 were observed. These data are consistent with a recent report showing that the phosphorylation of some Akt substrates is independent of the phosphorylation state of Ser473 (Jacinto et al., 2006). In particular, the phosphorylation state of FoxO1 on Ser256 was reported to be the same in normal cells and in SIN1 knockdown cells (where Ser473 phosphorylation is abolished) (Jacinto et al., 2006). The possibility that the species of Akt phosphorylated at Thr308, but not Ser473, has activity toward some substrates adds a new level of fine-tuning to signal control by the PHLPP isoforms. Lastly, we found some substrates that displayed intermediate specificity. The phosphorylation state of TSC2 was affected by a PHLPP2/Akt1 and by PHLPP1/Akt2 signaling complexes but was relatively insensitive to Akt3. These data underscore the role of isoform specificity in driving downstream signaling of the PI3K/Akt pathway.

Feedback Regulation of Akt Signaling Activated by Depletion of Both PHLPP Isoforms

Our data also reveal that the simultaneous knockdown of both PHLPP1 and PHLPP2 activates feedback regulation of Akt mediated by p70S6K (Harrington et al., 2004; O'Reilly et al., 2006; Um et al., 2004). Thus, although PHLPP1 and PHLPP2 control different Akt isozymes, depletion of both isoforms does not enhance Akt phosphorylation relative to single knockdown because, in cells lacking both PHLPP isoforms, Akt phosphorylation is

inhibited by activation of the p70S6K-mediated feedback loop. This feedback inhibition is suppressed by rapamycin, allowing additivity in the effects of knockdown of PHLPP1 and PHLPP2. These results underscore the importance of compensatory mechanisms that constrain perturbations to signaling pathways.

Summary

Our data reveal that PHLPP1 and PHLPP2 selectively dephosphorylate specific Akt isoforms, thus differentially controlling the amplitude of Akt signaling. Taken together with results from Akt knockout mice, it is intriguing to suggest that PHLPP1 plays a role in glucose homeostasis (where Akt2 is critical) whereas PHLPP2 plays a role in cell survival (where Akt1 is critical) (Chen et al., 2001). Our data also underscore the role of spatial segregation (i.e., via specific signaling complexes such as PHLPP1:Akt2 and PHLPP2:Akt1) in driving specificity in both signal propagation and signal termination in the PI3K/Akt pathway.

EXPERIMENTAL PROCEDURES

PHLPP2-specific siRNA was purchased from Dharmacon and targeted the following sequences: 5′-CCTAAGTGGCAACAAGCTT-3′ (si-1); 5′-CCATTCAAGATGAGTTGCT-3′ (si-2); and 5′-GGACAGCCT GAACCTCATTG-3′ (si-3) in PHLPP2. SMARTpool siRNA (combining all three siRNAs) was used for all experiments except Figure 1C, which used si-1.) SMARTpool siRNA against PHLPP1 and PHLPP2 was also purchased from Dharmacon. Akt isoform-specific siRNA was purchased from Santa Cruz.

Cloning and Expression

Full-length PHLPP2 cDNA was cloned by combining the bf979574 cDNA (I.M.A.G.E. Consortium; sequencing revealed the C terminus of construct was same as BC035267 cDNA) and AB023148 cDNA (Kazusa cDNA collection). Five nucleotides were not present in bf979574 cDNA based on the predicted PubMed sequence, NM_015020, and these nucleotides were added using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The nucleotide sequence of the resulting two constructs (bf979574 and AB023148) corresponded to the predicted sequence NM_015020, resulting in the amino acid sequence in Figure 1A. Because of a discrepancy in the amino acid at position 542 in the predicted sequence of NM_015020 (Val) with that of Sequence ID Q6ZVD8 (Leu), we sequenced seven cell lines (H157, 293T, MDA-MB-231, ZR-75-1, MCF-7, SK-BR-3, and T47D) and confirmed that Leu is the correct residue. To express HA-tagged full-length PHLPP2, sequence was amplified by PCR and subcloned into NotI and XbaI sites in the pcDNA3HA vector (Gao et al., 2005). A GST-tagged construct of the PP2C domain for bacterial expression was generated by amplifying the coding region of the PP2C domain (corresponding to residues 780–1030) by PCR and subcloning the sequence into EcoRI and XhoI sites of pGEX-KG vector (Hakes and Dixon, 1992).

Cell Transfections and Immunoblotting

ZR-75-1 and SKBR3 cell lines were maintained in RPMI 1640 (Cellgro), and all other cell lines were maintained in DMEM (Cellgro); both media were supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were maintained at 37°C in 5% CO_2. Transient transfections and siRNA experiments were performed as previously described (Gao et al., 2005), except for ZR-75-1 cells, which were transfected using FuGENE6 reagent (Roche). Transfection efficiencies (determined by gating cells transfected with GFP using flow cytometry) for 293T and H157 cell lines averaged between 70% and 90% for each experiment; efficiencies for breast cancer cell lines averaged between 50% and 85%. For immunoblotting, transfected cells were lysed in buffer 1 (50 mM Na_2HPO_4 [pH 7.5], 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% SDS, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 1 mM PMSF) and sonicated for 5 s. Lysates containing equal protein were analyzed on SDS-PAGE gels, and individual blots were probed using the indicated antibody. Densitometric analysis was performed with the NIH Image analysis software (version 1.63).

Phosphatase Assays and Coimmunoprecipitations

GST-PP2C was expressed in BL21 bacterial cells; phosphatase assays using *p*NPP as substrate were performed using the purified GST-PP2C construct in buffer containing 0.05 M Tris, 0.05 M Bis-Tris, and 0.1 M sodium acetate (pH 7.5) at 28°C. Dephosphorylation of *p*NPP was measured by continuously monitoring the change in absorbance at 405 nM using a Thermo Electron Corp. Genesys 10 UV-visible spectrophotometer. Initial rates were determined using the molar extinction coefficient of 12.8 $mM^{-1}cm^{-1}$ for the product *para*-nitrophenol (*p*NP) at pH 7.5. To determine the kinetic parameters k_{cat} and k_{cat}/K_m, the initial velocities were measured at various substrate concentrations and the data were fit to the Michaelis-Menten equation by nonlinear regression analysis. Phosphatase assays were also performed using the GST-PP2C construct conjugated to glutathione-Sepharose and pure Akt1 as a substrate as previously described (Gao et al., 2005). The activity of full-length HA-PHLPP2 was assessed by expressing and immunoprecipitating PHLPP2 from 293T or H157 cell lysates. Cells were lysed in buffer 2 (20 mM HEPES [pH 7.4], 1% Triton X-100, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 1 mM PMSF). Detergent-soluble lysates were incubated overnight at 4°C with HA antibody and ultra-link protein A/G beads (Pierce). Beads were then washed three times with buffer 1 and incubated in phosphatase buffer with purified phosphorylated Akt as previously described (Gao et al., 2005). HA-PHLPP2 was immunoprecipitated from 293T cells as described above and the cells were lysed in buffer 3 (50 mM Na_2HPO_4 [pH 7.5], 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, and 1 mM PMSF) and washed in buffer 3 four times. Akt agarose was used to immunoprecipitate endogenous Akt (Upstate Biotechnologies). Akt isoforms were immunoprecipitated from H157 cells as described above using isoform-specific antibodies (Cell Signaling), washed four times in buffer 3, and probed with pan- or phospho-specific antibodies as well as PHLPP1- or PHLPP2-specific antibodies from Bethyl Laboratories.

In Vitro Kinase Assay

Akt was immunoprecipitated from cell lysates using Akt agarose, and kinase reactions were performed using an Akt kinase assay kit (Cell Signaling) as described previously (Brognard et al., 2001).

Immunofluorescence Staining

Hs578Bst and H157 cells were seeded onto glass coverslips and allowed to attach for ~24 hr. Cells were treated with control or isoform-specific PHLPP siRNA for 48 hr under high serum conditions. Cells were washed with PBS and fixed in 3% paraformaldehyde and 2% sucrose for 15 min at room temperature. Fixed cells were washed in PBS and quenched in 0.1% glycine for 5 min at room temperature. Cells were then permeabilized in 0.1% Triton X-100 for 15 min at room temperature, washed in PBS, and exposed to blocking buffer (50% FBS in PBS) for 15 min at room temperature. Cells were incubated in primary antibody (1:1000) in 10% FBS PBS overnight at 4°C. Cells were then washed three times in PBS and incubated in secondary antibody (Alexa Fluor 488 goat antirabbit IgG [1:500 in 10% FBS in PBS]), washed an additional three times, and coverslips were mounted onto

slides with VectorShield and viewed using a Zeiss Axiovert 200 microscope.

Proliferation and Apoptosis Assays

Apoptotic assays were performed as previously described (Gao et al., 2005). For breast cancer cell lines, apoptotic assays were performed on whole cell population and not gated cells. To determine G1/S ratios, cells were cotransfected with GFP and HA-PHLPP2 and incubated with high serum (10% FBS) for 48 hr; cells were gated based on GFP expression as described previously (Gao et al., 2005). For BrdU incorporation assays, cells were maintained in high serum growth media (10% FBS) and transfected with 100nM SMARTpool siRNA and incubated for 48 hr prior to performing assays following manufacturer's protocol (Oncogene Research Products).

Statistical Analysis

Statistical analysis was performed using JMP 5.1 statistical software (SAS Institute Inc). The significance of differences between siRNA control and siPHLPP1, siPHLPP2, or both was determined using one-way ANOVA statistical analysis followed by posthoc Student's t tests.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and can be found with this article online at http://www.molecule.org/cgi/content/full/25/6/917/DC1/.

ACKNOWLEDGMENTS

We thank Dr. Phillip Dennis for the H157 cell line. We thank Drs. Tony Hunter, Jack Dixon, Steve Dowdy, and Tony Wynshaw-Boris for helpful discussions. We thank Dr. Mark Lawson for guidance and help with statistical analysis. We thank Dr. Maya Kunkel for purified Akt used in phosphatase assays. DNA sequencing was performed by the DNA Sequencing Shared Resource, UCSD Cancer Center, which is funded in part by NCI Cancer Center Support Grant #2 P30 CA23100-18. This work was supported by DOD BCRP Predoctoral Grant BC043239 (J.B.), pharmacology training grant 5 T32 GM07752-26 (J.B.), and National Institutes of Health Grant K01CA10209 (T.G.) and GM067946 (A.C.N.). Regarding grant BC043239, the U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014, is the awarding and administering acquisition office. The content of this article does not necessarily reflect the position or the policy of the U.S. government.

Received: February 17, 2006
Revised: August 30, 2006
Accepted: February 20, 2007
Published: March 22, 2007

REFERENCES

Altomare, D.A., and Testa, J.R. (2005). Perturbations of the AKT signaling pathway in human cancer. Oncogene *24*, 7455–7464.

Andjelkovic, M., Jakubowicz, T., Cron, P., Ming, X.F., Han, J.W., and Hemmings, B.A. (1996). Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. Proc. Natl. Acad. Sci. USA *93*, 5699–5704.

Biggs, W.H., 3rd, Meisenhelder, J., Hunter, T., Cavenee, W.K., and Arden, K.C. (1999). Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc. Natl. Acad. Sci. USA *96*, 7421–7426.

Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M., and Alessi, D.R. (2001). The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. EMBO J. *20*, 4380–4390.

Bornancin, F., and Parker, P.J. (1997). Phosphorylation of protein kinase C-alpha on serine 657 controls the accumulation of active enzyme and contributes to its phosphatase-resistant state. J. Biol. Chem. *272*, 3544–3549.

Brazil, D.P., and Hemmings, B.A. (2001). Ten years of protein kinase B signaling: A hard Akt to follow. Trends Biochem. Sci. *26*, 657–664.

Brognard, J., Clark, A.S., Ni, Y., and Dennis, P.A. (2001). Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res. *61*, 3986–3997.

Chen, W.S., Xu, P.Z., Gottlob, K., Chen, M.L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., et al. (2001). Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev. *15*, 2203–2208.

Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw, E.B., 3rd, Kaestner, K.H., Bartolomei, M.S., Shulman, G.I., and Birnbaum, M.J. (2001a). Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science *292*, 1728–1731.

Cho, H., Thorvaldsen, J.L., Chu, Q., Feng, F., and Birnbaum, M.J. (2001b). Akt1/PKBalpha is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. J. Biol. Chem. *276*, 38349–38352.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231–241.

Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: A play in three Akts. Genes Dev. *13*, 2905–2927.

Edwards, A.S., and Newton, A.C. (1997). Phosphorylation at conserved carboxyl-terminal hydrophobic motif regulates the catalytic and regulatory domains of protein kinase C. J. Biol. Chem. *272*, 18382–18390.

Ferguson, K.M., Kavran, J.M., Sankaran, V.G., Fournier, E., Isakoff, S.J., Skolnik, E.Y., and Lemmon, M.A. (2000). Structural basis for discrimination of 3-phosphoinositides by pleckstrin homology domains. Mol. Cell *6*, 373–384.

Gao, T., Furnari, F., and Newton, A.C. (2005). PHLPP: A phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol. Cell *18*, 13–24.

Hakes, D.J., and Dixon, J.E. (1992). New vectors for high-level expression of recombinant proteins in bacteria. Anal. Biochem. *202*, 293–298.

Harrington, L.S., Findlay, G.M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N.R., Cheng, S., Shepherd, P.R., et al. (2004). The TSC1–2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J. Cell Biol. *166*, 213–223.

Huang, X., Begley, M., Morgenstern, K.A., Gu, Y., Rose, P., Zhao, H., and Zhu, X. (2003). Crystal structure of an inactive Akt2 kinase domain. Structure *11*, 21–30.

Irie, H.Y., Pearline, R.V., Grueneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S., and Brugge, J.S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J. Cell Biol. *171*, 1023–1034.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell *127*, 125–137.

Jackson, M.D., Fjeld, C.C., and Denu, J.M. (2003). Probing the function of conserved residues in the serine/threonine phosphatase PP2Calpha. Biochemistry *42*, 8513–8521.

Jiang, Z.Y., Zhou, Q.L., Coleman, K.A., Chouinard, M., Boese, Q., and Czech, M.P. (2003). Insulin signaling through Akt/protein kinase B analyzed by small interfering RNA-mediated gene silencing. Proc. Natl. Acad. Sci. USA *100*, 7569–7574.

Jope, R.S., and Johnson, G.V. (2004). The glamour and gloom of glycogen synthase kinase-3. Trends Biochem. Sci. *29*, 95–102.

Kunkel, M.T., Ni, Q., Tsien, R.Y., Zhang, J., and Newton, A.C. (2005). Spatio-temporal dynamics of protein kinase B/Akt signaling revealed by a genetically encoded fluorescent reporter. J. Biol. Chem. *280*, 5581–5587.

Kwiatkowski, D.J., and Manning, B.D. (2005). Tuberous sclerosis: A GAP at the crossroads of multiple signaling pathways. Hum Mol Genet *14*, R251–R258.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., et al. (1997). PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science *275*, 1943–1947.

Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *273*, 13375–13378.

Marygold, S.J., and Leevers, S.J. (2002). Growth signaling: TSC takes its place. Curr. Biol. *12*, R785–R787.

Mayo, L.D., and Donner, D.B. (2001). A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. Proc. Natl. Acad. Sci. USA *98*, 11598–11603.

O'Reilly, K.E., Rojo, F., She, Q.B., Solit, D., Mills, G.B., Smith, D., Lane, H., Hofmann, F., Hicklin, D.J., Ludwig, D.L., et al. (2006). mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. *66*, 1500–1508.

Pap, M., and Cooper, G.M. (1998). Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. J. Biol. Chem. *273*, 19929–19932.

Pap, M., and Cooper, G.M. (2002). Role of translation initiation factor 2B in control of cell survival by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta signaling pathway. Mol. Cell. Biol. *22*, 578–586.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S.M., Riggins, G.J., et al. (2004). High frequency of mutations of the PIK3CA gene in human cancers. Science *304*, 554.

Sarbassov, D.D., Guertin, D.A., Ali, S.M., and Sabatini, D.M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science *307*, 1098–1101.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L., and Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol. Cell *22*, 159–168.

Shimizu, K., Okada, M., Nagai, K., and Fukada, Y. (2003). Suprachiasmatic nucleus circadian oscillatory protein, a novel binding partner of K-Ras in the membrane rafts, negatively regulates MAPK pathway. J. Biol. Chem. *278*, 14920–14925.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J., and Thomas, G. (2004). Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature *431*, 200–205.

Viglietto, G., Motti, M.L., Bruni, P., Melillo, R.M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., et al. (2002). Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. Nat. Med. *8*, 1136–1144.

Vivanco, I., and Sawyers, C.L. (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat. Rev. Cancer *2*, 489–501.

Yang, J., Cron, P., Thompson, V., Good, V.M., Hess, D., Hemmings, B.A., and Barford, D. (2002). Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. Mol. Cell *9*, 1227–1240.

Yoeli-Lerner, M., Yiu, G.K., Rabinovitz, I., Erhardt, P., Jauliac, S., and Toker, A. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol. Cell *20*, 539–550.

Zhou, B.P., Liao, Y., Xia, W., Spohn, B., Lee, M.H., and Hung, M.C. (2001). Cytoplasmic localization of p21Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. Nat. Cell Biol. *3*, 245–252.

JBC Papers in Press. Published on December 27, 2007 as Manuscript M707319200
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M707319200

The phosphatase PHLPP controls the cellular levels of protein kinase C

Tianyan Gao[1], John Brognard[2], and Alexandra C. Newton
From the Department of Pharmacology, [2]Biomedical Sciences Graduate Program, University of California at San Diego, La Jolla, CA 92093-0721. [1]Present address: Department of Pharmacology and Toxicology, Sealy Center for Cancer Cell Biology, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1048
Running Title: PHLPP-mediated dephosphorylation of protein kinase C
Address correspondence to: Alexandra C. Newton, Department of Pharmacology, University of California San Diego, 9500 Gilman Dr. La Jolla, CA 92093-0721; Tel. (858) 534-4527; FAX (858) 822-5888; E-Mail: anewton@ucsd.edu

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 C-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. Lastly, 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 PHLPP (PH domain Leucine-rich repeat Protein Phosphatase) 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

(S473 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 C-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 C-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 switches (activation loop and C-terminus) are conserved amongst 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 RNAi 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; phospho-antibodies for T641 (P641) and Ser660 (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 T500 (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 C-terminus (CT, amino acid residues 907-1205). Relevant regions were amplified using PCR and the PCR products were subcloned into *EcoR I* and *Xho I* sites on pcDNA3HA vector (2) or *Kpn I* and *Not I* sites on pEBG vector (20). Since expression of 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 (N-terminus plus pseudosubstrate), C1A (C1A domain), C1B (C1B domain), C1AB (C1A plus C1B domains), NC1 (N-terminus plus the entire C1 domain), C2 (C2 domain), KD (kinase domain), and CT (C-terminus) were constructed as described previously (20,21). The full-length human PP2Cα cDNA was cloned by one-step RT-PCR using human brain total RNA as template. The coding region of PP2Cα was amplified using PCR and subcloned into *Xho I* and *Xba I* 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 PBS containing 1% Triton X-100 and 10 mM imidazole, and His-PKC βII proteins were purified using Ni-NTA beads (Qiagen). The dephosphorylation reactions were carried out in a reaction buffer containing 50 mM Tris (pH 7.4), 1 mM DTT and 5 mM $MnCl_2$ at 30° C for 30 minutes. The final concentration of His-PKC βII and GST-PP2C in the reactions were 50 nM and 10 nM, respectively.

Cell transfection and siRNA targeting sequences

COS7, 293T, and H157 cells were maintained in DMEM (Cellgro) containing 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) TGATCTAGATGCTATGATT; 3) GATATTGGCCATAATCAAA; and 4) GAACGCCTCTGCGAACAAA; for PHLPP2, 1) GGAAAGACCCAGCTGCATA; 2) GAACTTGTCCCATAATAAA; 3) GCTATAATCTTCTCACAGA; and 4) GTACAGCAGTCAACTAATG. The final concentration of the SmartPool siRNAs used in

transfections was between 50-100 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 DTT, 200 μM benzamidine, 40 μg ml^{-1} leupeptin, 200 μM PMSF). The cells were lysed by brief sonication, and the cell lysates were subjected to centrifugation at 16,000 x *g* for 5 minutes at 4 ºC. 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-solublized 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-solublized cell lysates were incubated with glutathione-Sepharose at 4 ºC for 2 hours. 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 hours 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.

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 towards 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 all three conserved sites T500, T641, and S660 (17). The Western blot in Figure 1A shows that the PP2C domain effectively dephosphorylated the hydrophobic motif site, S660, and the turn motif, T641. In contrast, the activation loop site T500 was relatively resistant to dephosphorylation by the PP2C domain of PHLPP1 *in vitro*. These results 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 Figure 1B show the partitioning of PKC βII in the detergent-soluble supernant (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 C-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 (Figure 1B, 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 C-terminal site, are not detectable. These results reveal that co-expression of PHLPP1 with PKC βII in cells promotes 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 (T641) or the hydrophobic motif (S660) in the PHLPP-mediated distribution of PKC to the detergent-insoluble pellet, we examined two phosphomimetics of PKC βII: T641E and S660E. Figure 1C shows that the PHLPP-mediated redisitribution was not affected by negative charge on T641 but was prevented when negative charge was locked on S660 (quantified data shown in Figure 1D). These data suggest that loss of negative charge on S660, but not T641, 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). Figure 2A 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 (lane1) or HA-PP2Cα (lane 3). Note that the 'Input' blot in Figure 2A 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 Figure 2B 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 C-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 C-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 (Figure 2C). 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 Figures 1 and 3, co-expression of WT PHLPP1 with PKC βII resulted in the accumulation of a faster-migrating species of PKC βII (Figure 3A, dash) in the detergent-insoluble fraction of the cells (Figure 3A, lane 6) that was absent in vector co-transfected cells (Figure 3A, 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 C-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 necessary 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 (Figure 3B, lane 2, and Figure 3C). This finding is consistent with the PH domain of PHLPP1 providing a docking site for binding PKC βII as shown in Figure 2C. In contrast, deletion of the PDZ-binding motif had no significant effect on the interaction of PHLPP1 with PKC βII (Figure 3B, lane 3 and Figure 3C). 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 — Since there are two functionally related isoforms in the PHLPP family, we next addressed whether the second isoform, PHLPP2, also dephosphorylates PKC. Figure 4A shows that co-expression of HA-PHLPP2 increased the amount of dephosphorylated PKC βII in the detergent-insoluble fraction of the cells (lane 2). Furthermore, co-immunoprecipitation experiments showed that PKC βII specifically interacted with PHLPP2 in cells (Figure 4B, 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 C-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 hour or left untreated, and the cell lysates were analyzed for the dephosphorylation of endogenous PKC α. Figure 5A 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 (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 (T497 in PKC a) was not observed (data not shown, see Figure 5B and 5C as well). Note that an intermediate species (single asterisk) was detected by the phospho-specific antibody for phospho-T641, but not phospho-S660, indicating that dephosphorylation of S660 precedes that of T641. This is consistent with mass spectrometric analysis revealing that a minor species of PKC phosphorylated at T641 but not S660 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 (T500) 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, Figure 5B and the corresponding graph quantifying the data from three separate experiments (Figure 5C) show that treating cells with OA resulted in an approximately 2-fold increase in the phosphorylation at T500 for both WT and the S660E mutant of PKC βII compared to untreated control cells (P500 panels, lanes 1, 2, 5, and 6). Similarly, T500 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 T500 under the conditions of this experiment (lanes 3, and 7). These results are consistent with the dephosphorylation of the activation loop T500 site of PKC being catalyzed by an OA-sensitive phosphatase. Because the majority of WT PKC βII is fully-phosphorylated at both C-terminal sites basally (17), the phosphorylation of both the T641 and S660 sites in WT PKC βII was not affected by OA treatment alone (P641 and P660 panels, lanes 1 and 2; Figure 5C, 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 T641 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 T641 site. Figure 5C shows that the phosphorylation state of T641 in the S660E mutant increased 1.3-fold upon OA treatment compared to 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 approximately 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 approximately 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 T641 in the S660E mutant was similarly inhibited by OA pretreatment (Figure 5C, compare columns 19 and 20). These data establish that the dephosphorylation of T641, but not S660, is controlled by an OA-sensitive phosphatase. This insensitivity of the phosphorylation state of S660 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 Figure 6A 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 hours 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, approximately 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 (Figure 6B). In contrast, the PDBu treatment resulted in an approximately 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 of either PHLPP1 or PHLPP2 or both prevented this dephosphorylation (lanes 3-8, and Figure 6B). 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 the 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 hours. Figure 6C 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 T641 and S660 as well as the degradation of PKC was the same and approximately 1 hr. Genetic depletion of PHLPP1 and PHLPP2 had no significant effect on the loss of phosphate at T641: the half-time for dephosphorylation of this site was 0.9 ± 0.3 hr for the PHLPP knockdown compared to 0.7 ± 0.5 hrs for control) (Figure 6C, P641 panel; Figure 6D, P641 graph). However, the dephosphorylation of S660 was markedly reduced in cells depleted of PHLPP compared to control cells. Most noticeably, fitting the data to an exponential decay revealed that half as much PKC was dephosphorylated on S660 in cells lacking PHLPP compared to control cells: 44 ± 4% of the PKC was dephosphorylated in cells depleted of PHLPP compared to 82 ± 2% dephosphorylated in control cells (Figure 6D; note where curves plateau). The accumulation of an intermediate-migrating band (Figure 6C, labeled with single asterisk) in PHLPP knockkdown cells represented the large fraction of PKC phosphorylated at only S660 and not T641 (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 hrs in the knockdown cells compared to 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%) vs. fraction of PKC resistant to dephosphorylation on S660 (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 S660 but not T641 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 which has relatively high PHLPP expression, the

DLD-1 cell line. Figure 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 (Figure 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 to the control cells (Figure 7D, 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 (Figure 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 (S473) 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 (S660) 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 while 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. Since 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.

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 towards Akt: deletion of the last three C-terminal residues encoding the PDZ ligand inhibits the ability of PHLPP1 to dephosphorylate Akt, promote apoptosis, and suppress tumors. In contrast, deletion of the PH domain resulted in more robust phosphatase activity towards 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 towards 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 to 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 ester treatment. It has been reported that dephosphorylation and down-regulation of PKC occurs in cells following sustained elevation of the endogenous activator diacylglycerol (DAG) (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, since the endogenous activators of PKC, DAG 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 (Figure 8). First, we show that dephosphorylation of S660 precedes that of T641. Second, we show that depletion of PHLPP results in the accumulation of a species of PKC that is phosphorylated on S660, but not T641, that is relatively resistant to degradation. Under the conditions of our experiments, approximately 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 S660.

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 (S473) (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 upregulated by silencing the expression of endogenous PHLPP using RNAi. The levels of PKC α and PKC βII increased approximately 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 Figures 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 to 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.

References

1. Hanahan, D., and Weinberg, R. A. (2000) *Cell* **100**(1), 57-70
2. Gao, T., Furnari, F., and Newton, A. C. (2005) *Mol Cell* **18**(1), 13-24
3. Brognard, J., Sierecki, E., Gao, T., and Newton, A. C. (2007) *Mol Cell* **25**(6), 917-931
4. Newton, A. C. (2003) *Biochem J* **370**(Pt 2), 361-371
5. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R. J., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P. T. (1998) *Science* **279**(5351), 710-714

6. Alessi, D. R., Kozlowski, M. T., Weng, Q. P., Morrice, N., and Avruch, J. (1998) *Curr Biol* **8**(2), 69-81
7. Newton, A. C., and Koshland, D. E., Jr. (1987) *J. Biol. Chem.* **262**, 10185-10188
8. Behn-Krappa, A., and Newton, A. C. (1999) *Curr Biol* **9**(14), 728-737
9. Newton, A. C. (2001) *Chem. Rev.* **101**, 2353-2364
10. Bornancin, F., and Parker, P. J. (1997) *J Biol Chem* **272**(6), 3544-3549
11. Hill, M. M., Andjelkovic, M., Brazil, D. P., Ferrari, S., Fabbro, D., and Hemmings, B. A. (2001) *J Biol Chem* **276**(28), 25643-25646.
12. Parker, P. J., and Parkinson, S. J. (2001) *Biochem Soc Trans* **29**(Pt 6), 860-863.
13. Gysin, S., and Imber, R. (1996) *Eur J Biochem* **240**(3), 747-750
14. Hansra, G., Garcia-Paramio, P., Prevostel, C., Whelan, R. D., Bornancin, F., and Parker, P. J. (1999) *Biochem J* **342**(Pt 2), 337-344
15. Leontieva, O. V., and Black, J. D. (2004) *J Biol Chem* **279**(7), 5788-5801
16. Gysin, S., and Imber, R. (1997) *Eur J Biochem* **249**(1), 156-160
17. Keranen, L. M., Dutil, E. M., and Newton, A. C. (1995) *Curr Biol* **5**(12), 1394-1403
18. Hansra, G., Bornancin, F., Whelan, R., Hemmings, B. A., and Parker, P. J. (1996) *J. Biol. Chem.* **271**, 32785-32788
19. Dutil, E. M., Toker, A., and Newton, A. C. (1998) *Curr Biol* **8**(25), 1366-1375
20. Gao, T., Toker, A., and Newton, A. C. (2001) *J Biol Chem* **276**(22), 19588-19596
21. Chen, D., Purohit, A., Halilovic, E., Doxsey, S. J., and Newton, A. C. (2003) *J Biol Chem*
22. Dutil, E. M., Keranen, L. M., DePaoli-Roach, A. A., and Newton, A. C. (1994) *J Biol Chem* **269**(47), 29359-29362
23. Cohen, P. T. (2002) *J Cell Sci* **115**(Pt 2), 241-256
24. Borner, C., Eppenberger, U., Wyss, R., and Fabbro, D. (1988) *Proc Natl Acad Sci U S A* **85**(7), 2110-2114
25. Cohen, P., and Cohen, P. T. (1989) *J Biol Chem* **264**(36), 21435-21438
26. Edwards, A. S., and Newton, A. C. (1997) *J Biol Chem* **272**(29), 18382-18390
27. Gokmen-Polar, Y., Murray, N. R., Velasco, M. A., Gatalica, Z., and Fields, A. P. (2001) *Cancer Res* **61**(4), 1375-1381.
28. Murray, N. R., Davidson, L. A., Chapkin, R. S., Clay Gustafson, W., Schattenberg, D. G., and Fields, A. P. (1999) *J Cell Biol* **145**(4), 699-711
29. Balendran, A., Hare, G. R., Kieloch, A., Williams, M. R., and Alessi, D. R. (2000) *FEBS Lett* **484**(3), 217-223
30. Griner, E. M., and Kazanietz, M. G. (2007) *Nature Reviews. Cancer* **7**(4), 281-294
31. Kiley, S. C., Parker, P. J., Fabbro, D., and Jaken, S. (1991) *J Biol Chem* **266**(35), 23761-23768
32. Lee, H. W., Smith, L., Pettit, G. R., and Bingham Smith, J. (1996) *Am J Physiol* **271**(1 Pt 1), C304-311
33. Murray, N. R., Baumgardner, G. P., Burns, D. J., and Fields, A. P. (1993) *J Biol Chem* **268**(21), 15847-15853

ACKNOWLEDGEMENTS

We thank Lisa Gallegos for assistance with kinetic analysis of the data. This work was supported in part by National Institutes of Health R01 GM43154 (ACN), GM067946 (ACN), NIH K01 CA10209-03 (TG), and DOD BCRP Predoctoral Grant BC043239 (JB). Regarding grant BC043239 the U.S. Army Medical

Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office. The content of this article does not necessarily reflect the position or the policy of the U.S. Government.

The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GST, glutathione S-transferase; PDBu, phorbol-12, 13-dibutyrate. PDK-1, phosphoinositide-dependent kinase 1; PHLPP, PH-domain leucine-rich repeat protein phosphatase; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C;

FIGURE LEGENDS

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 T500, T641 or S660 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 (-)), while 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 BioRad 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 ± SEM of 3 independent experiments.

Figure 2. **PHLPP1 interacts with its substrate PKC.** (A) Co-immunoprecipitation 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 the 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, 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 the 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-solublized 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 the 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).

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 BioRad 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 ± SEM of 3 independent experiments.

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 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-solublized 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).

Figure 5. **Dephosphorylation of the hydrophobic motif site of PKC upon phorbol ester treatment is okadaic acid (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 hour (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 C-terminal

phosphorylation sites) is labeled with one asterisk (∗), and the fully-phosphorylated species (phosphates on both C-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: DMSO (lanes 1 and 5), OA (200 nM) for 30 minutes (lanes 2 and 6), PDBu (400 nM) for 2 hours (lanes 3 and 7), or pretreatment with OA (200 nM) for 30 minutes then PDBu (400 nM) for an additional 2 hours (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 BioRad 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 ± SEM of 3 independent experiments.

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 DMSO (lanes 1, 3, 5, and 7) or 400 nM PDBu (lanes 2, 4, 6, and 8) for 2 hours. The detergent-solublized 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 ζproteins 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 si-RNA or PHLPP-specific siRNA from 4 independent experiments. Western blots as shown in (A) were scanned and quantified using a BioRad 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 ± SEM. 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 C-termianl sites, single asterisk denotes PKC phosphorylated at only one C-terminal site, and dash represents PKC that is not phosphorylated at either C-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 mono-exponential curve to obtain the half-time and fraction of PKC dephosphorylated or degraded. Data represent the mean ± SEM (n=3).

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 ± SEM of 3 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.

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; T500 in PKC βII), the turn motif (orange circle; T641 in PKC βII) and the hydrophobic motif (green circle; S660 in PKC βII). This species of PKC is maintained in an inactive state by the autoinhibitory pseudosubstrate (green rectangle) which 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).

Figure 1

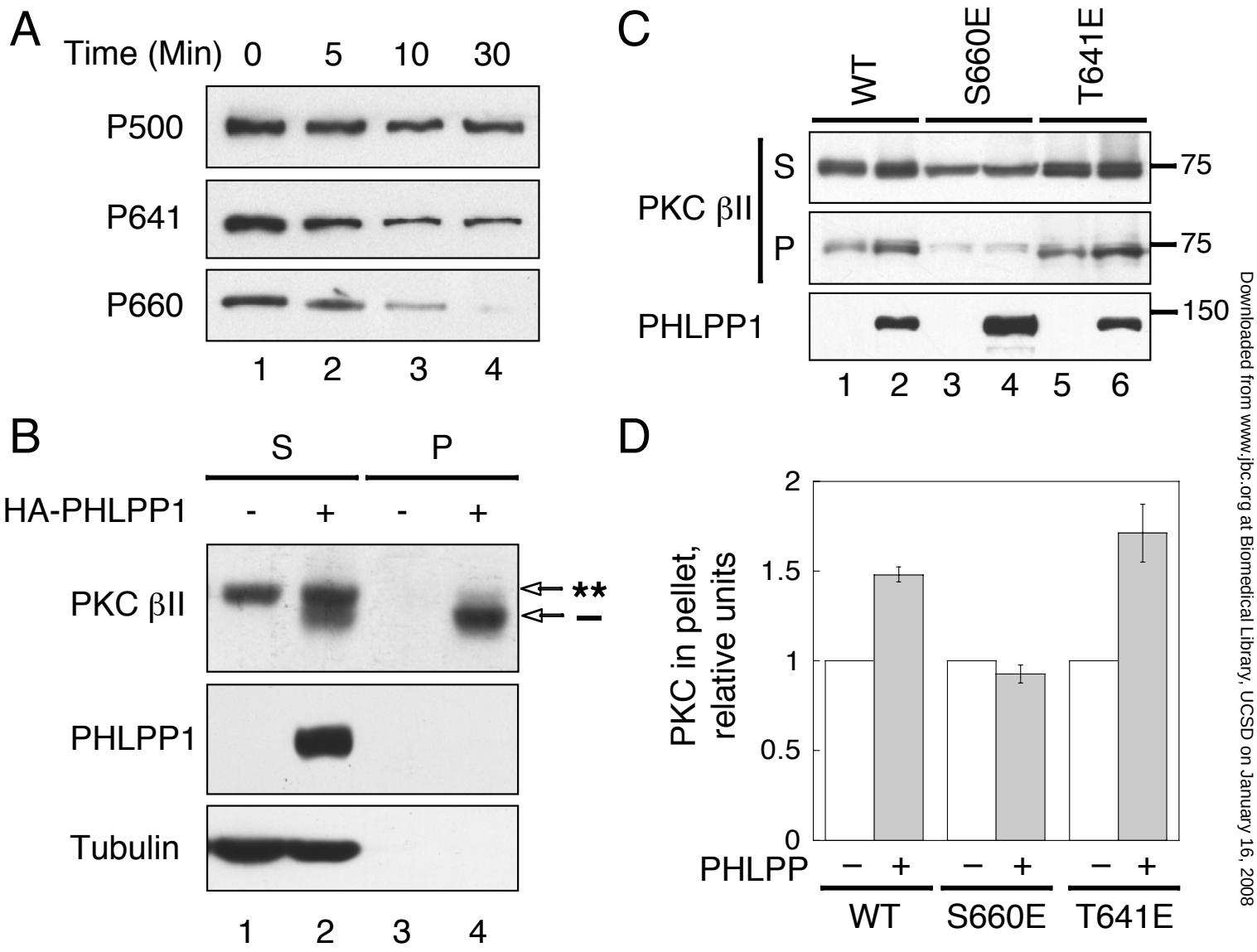

Figure 2

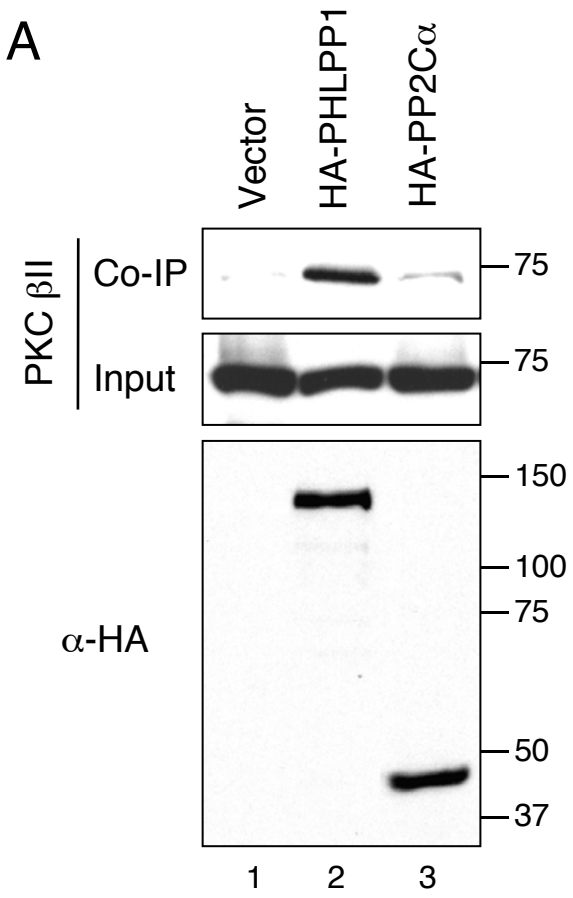

Figure 2

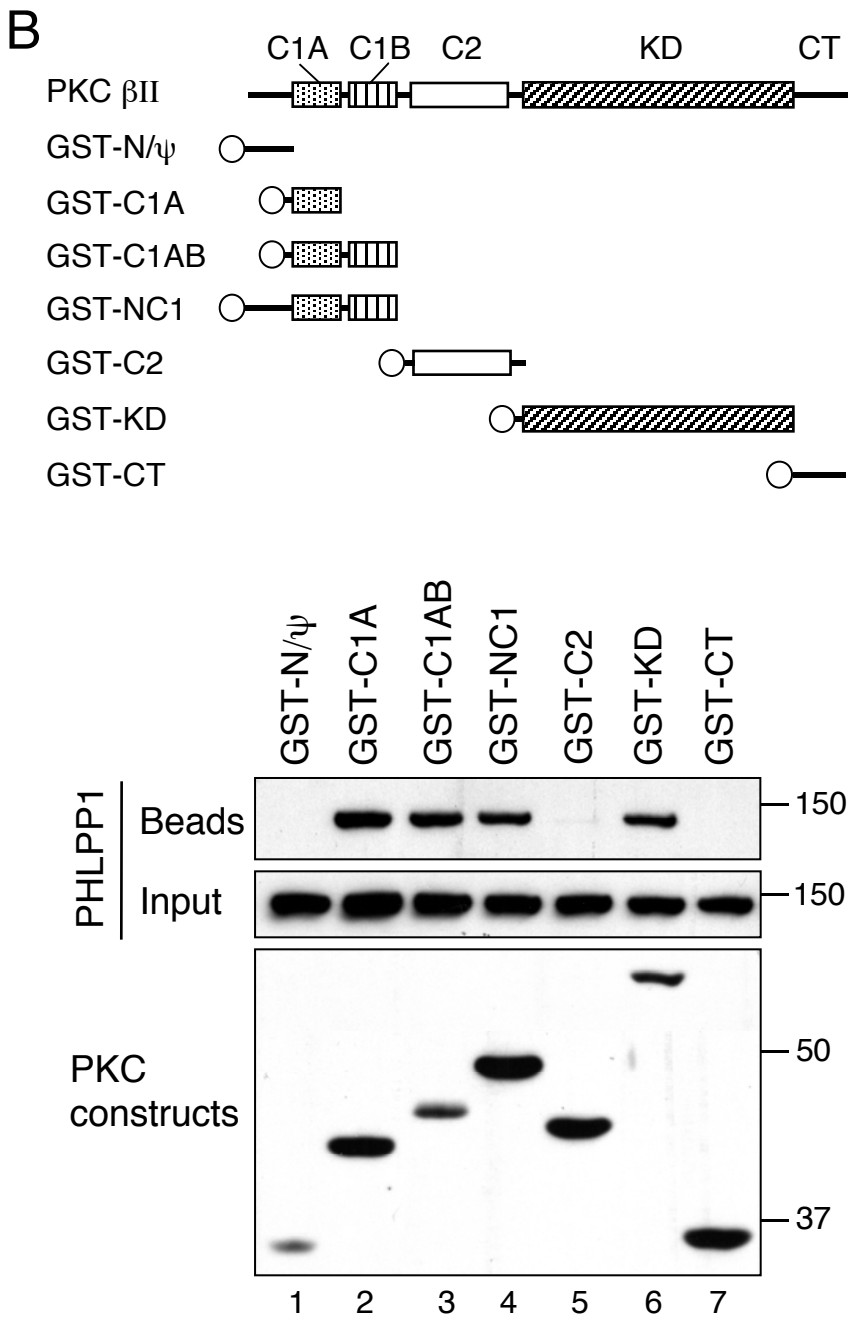

Figure 2

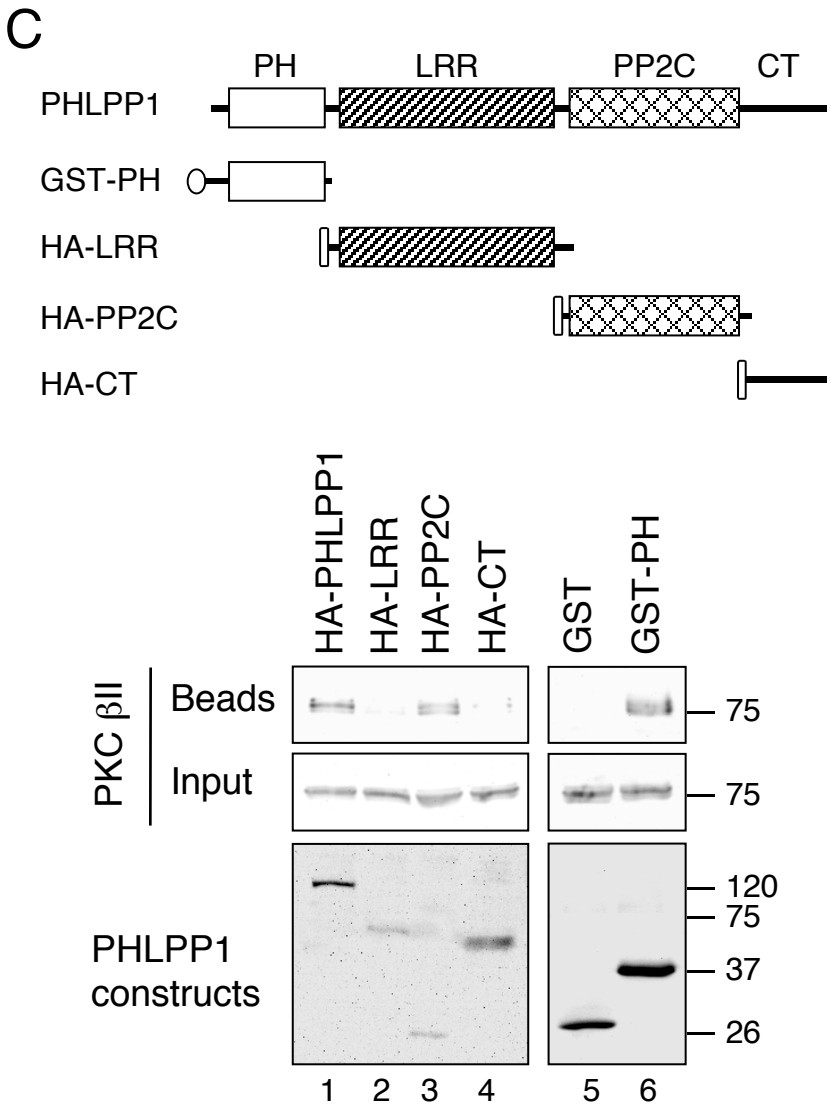

Figure 3

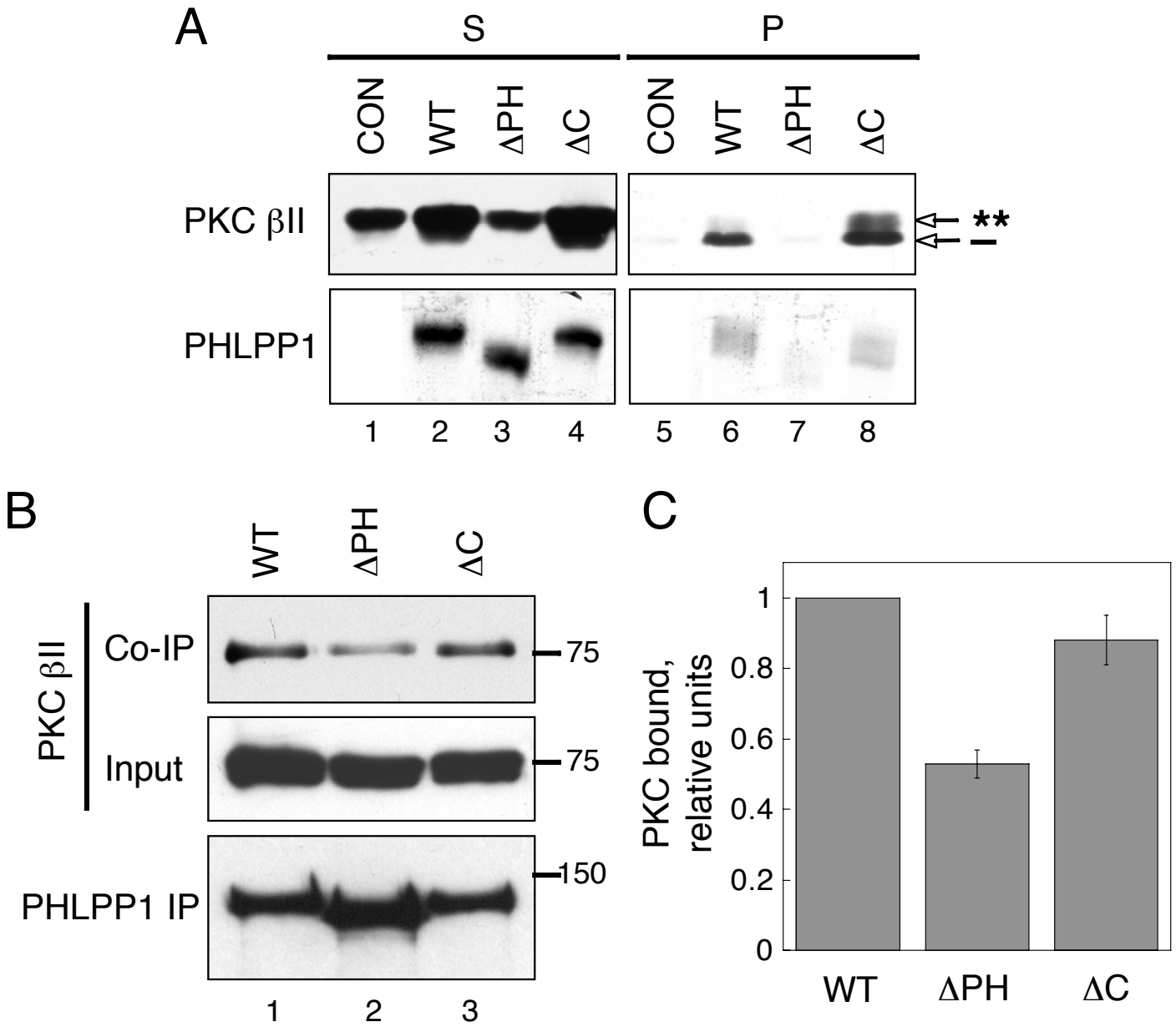

Figure 4

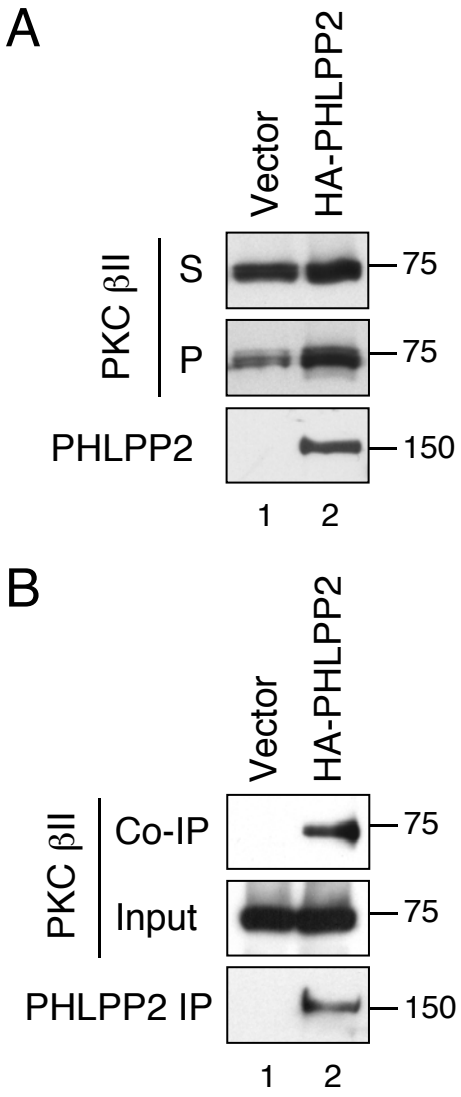

Figure 5

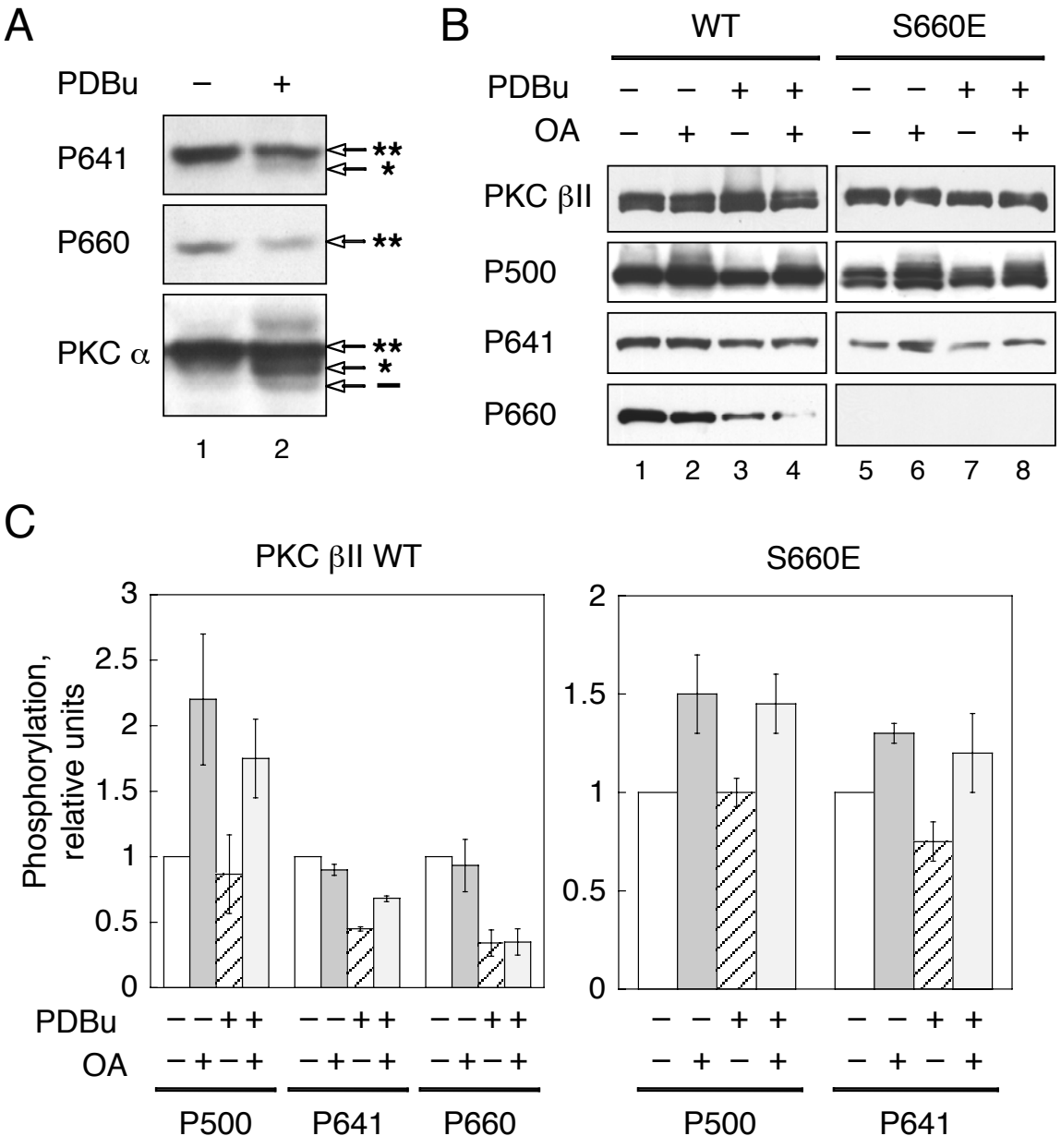

Figure 6

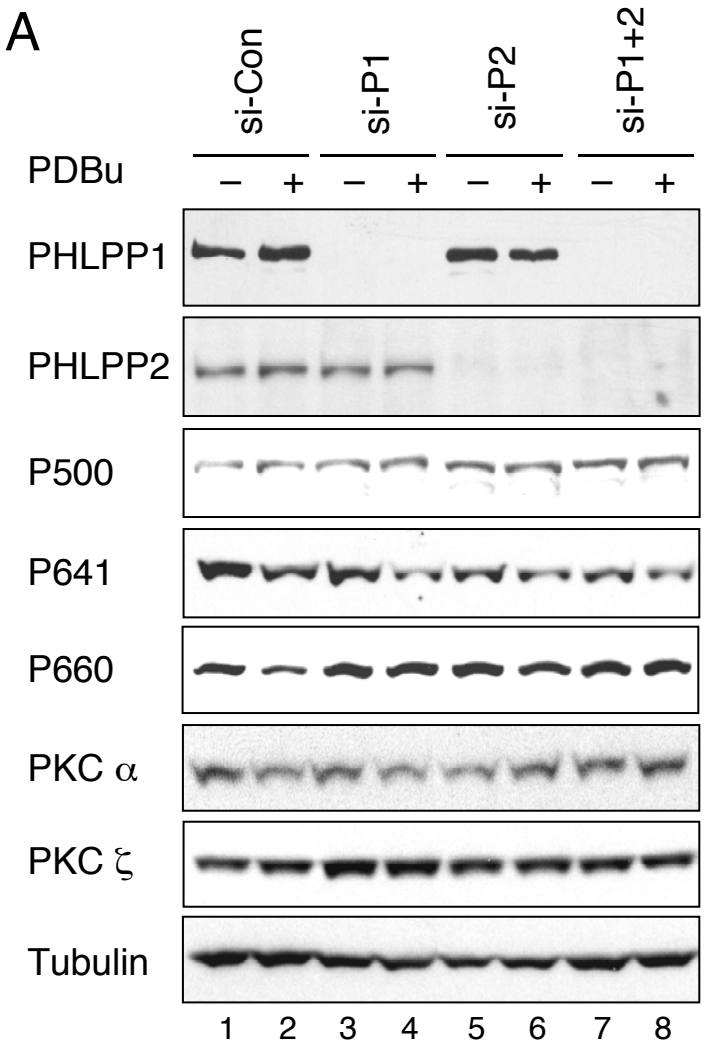

Figure 6

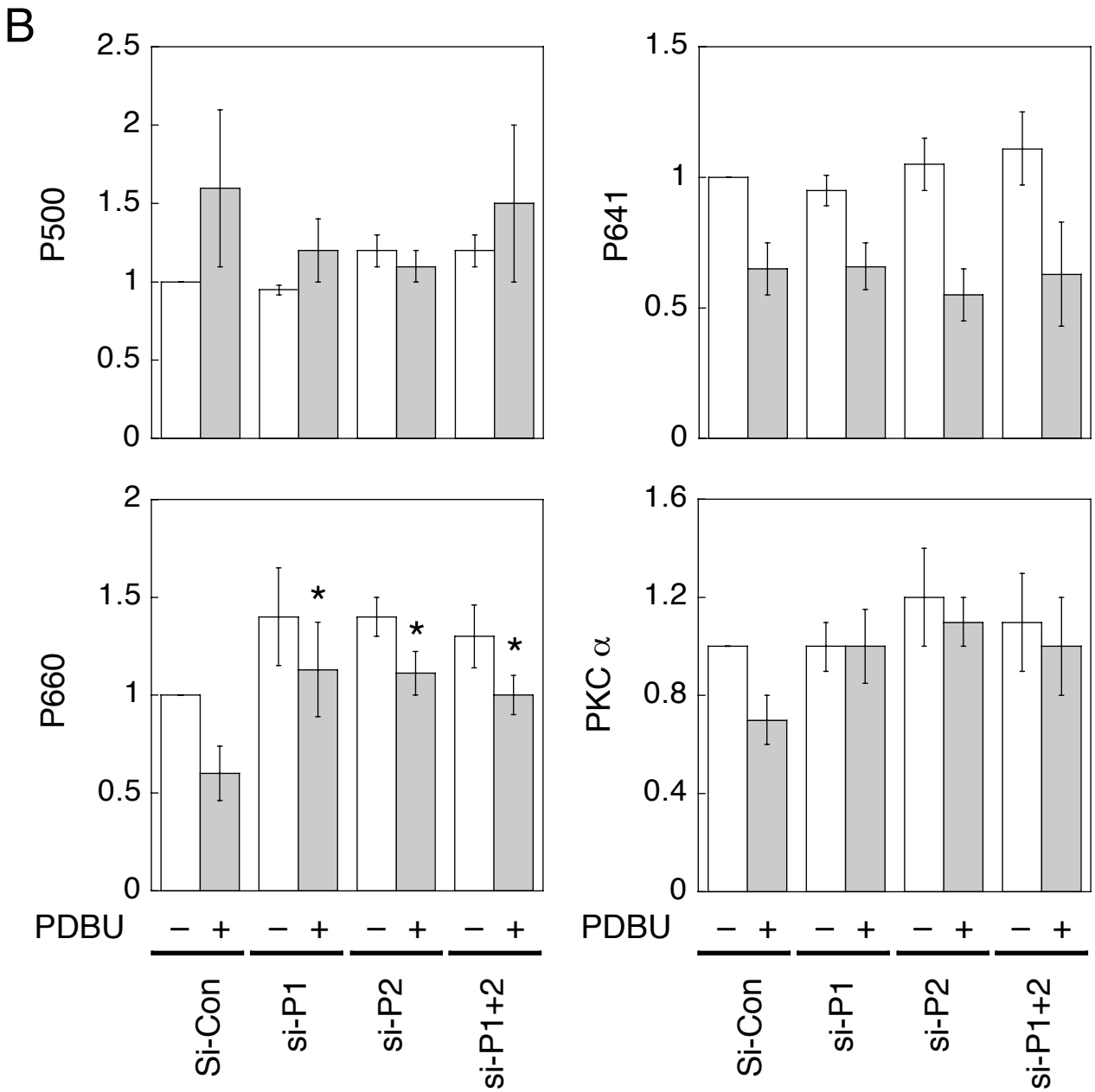

Figure 6

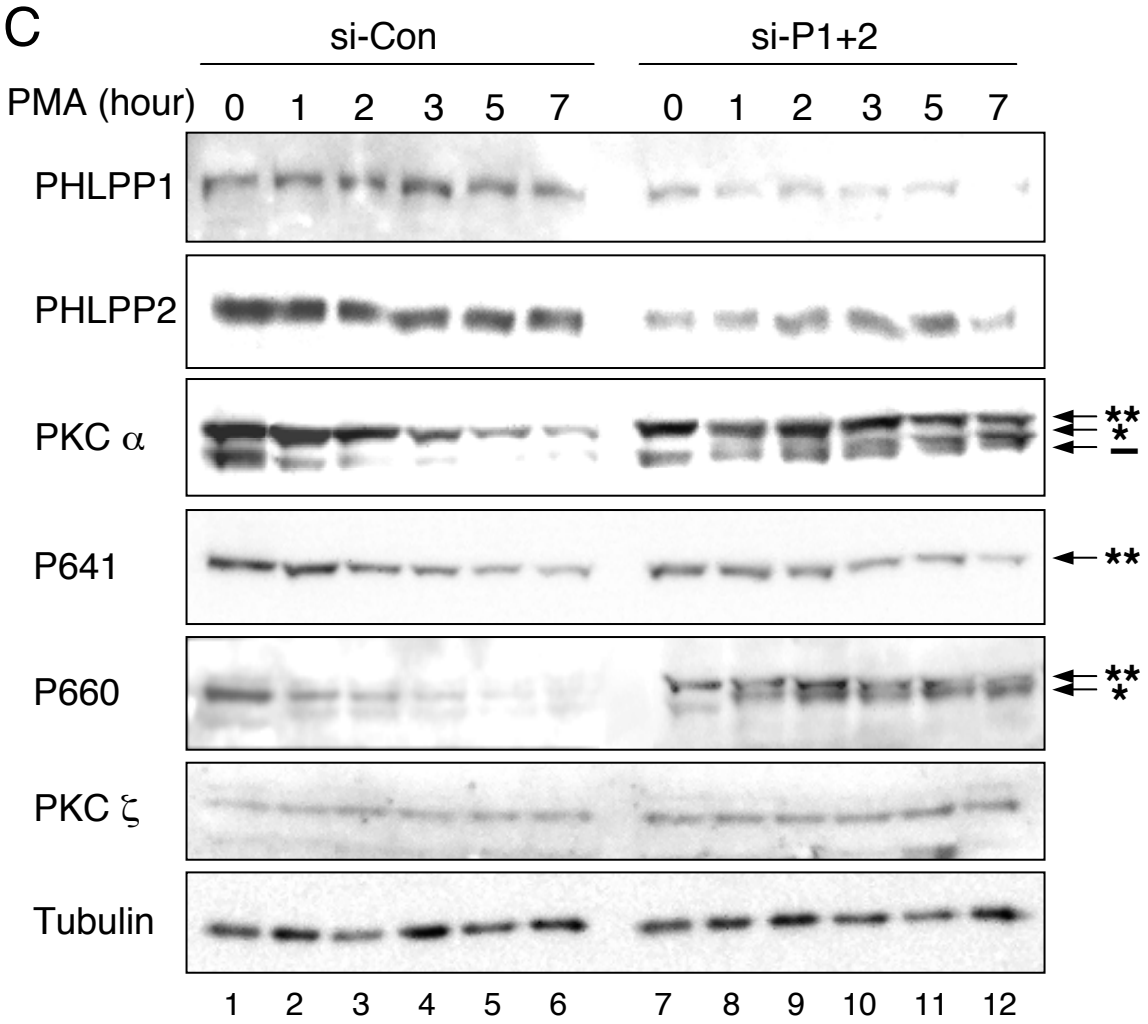

Figure 6

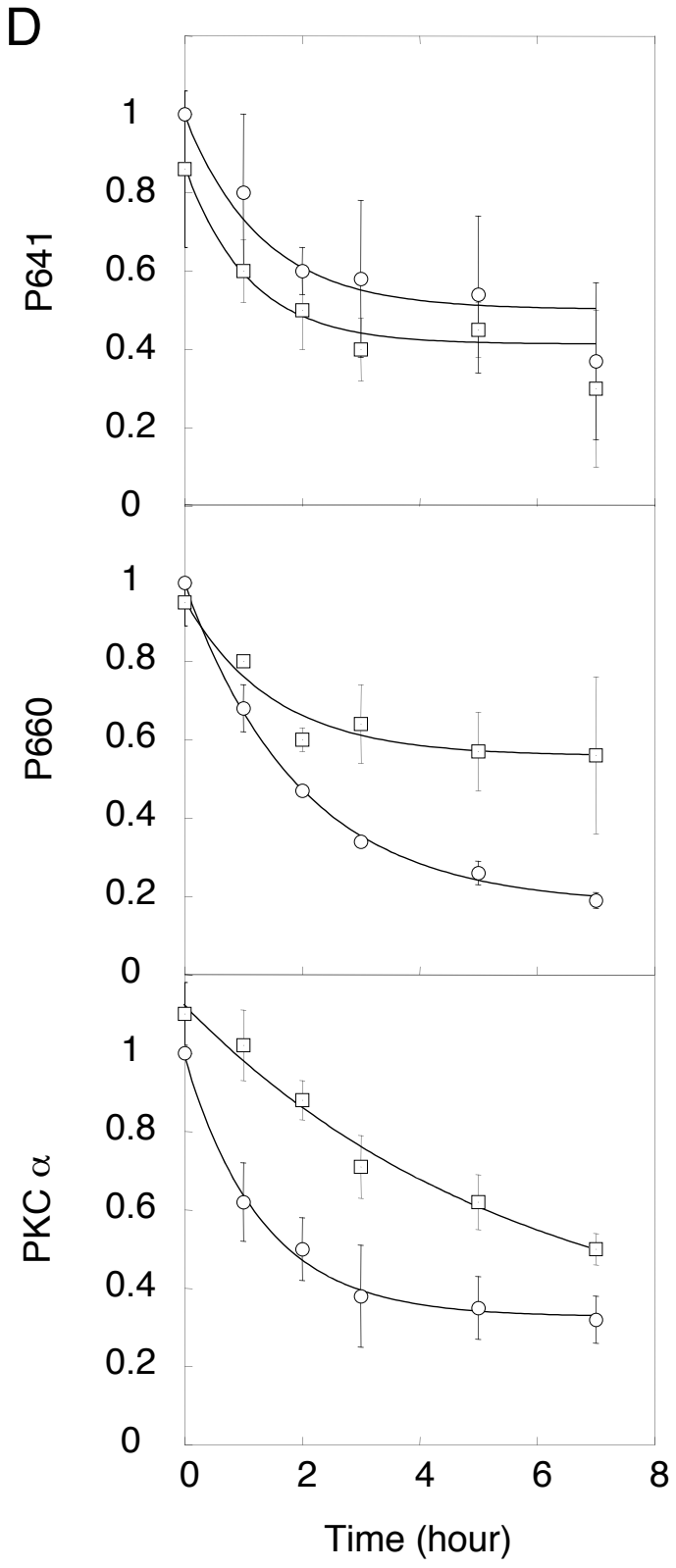

Figure 7

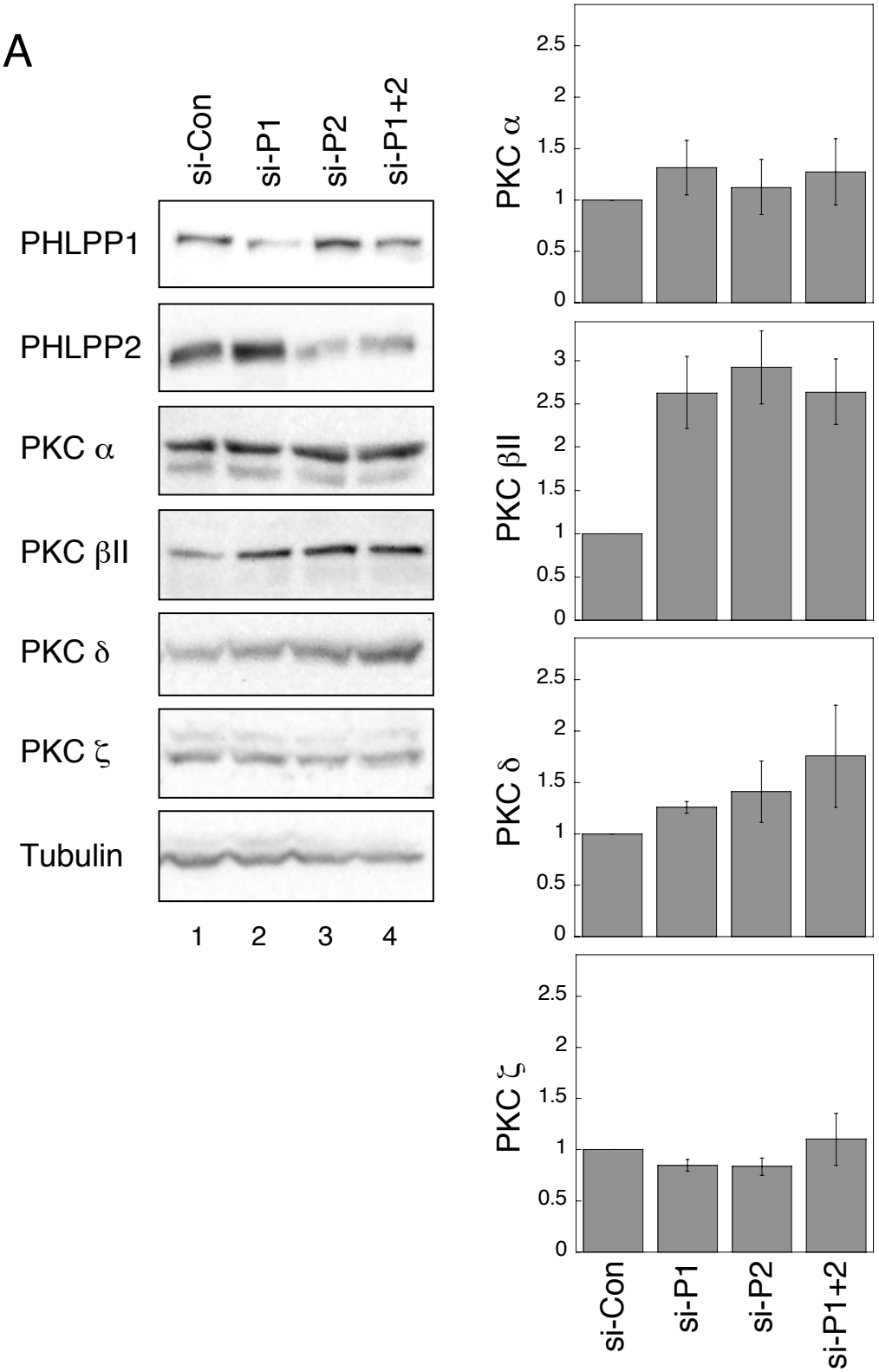

Figure 7

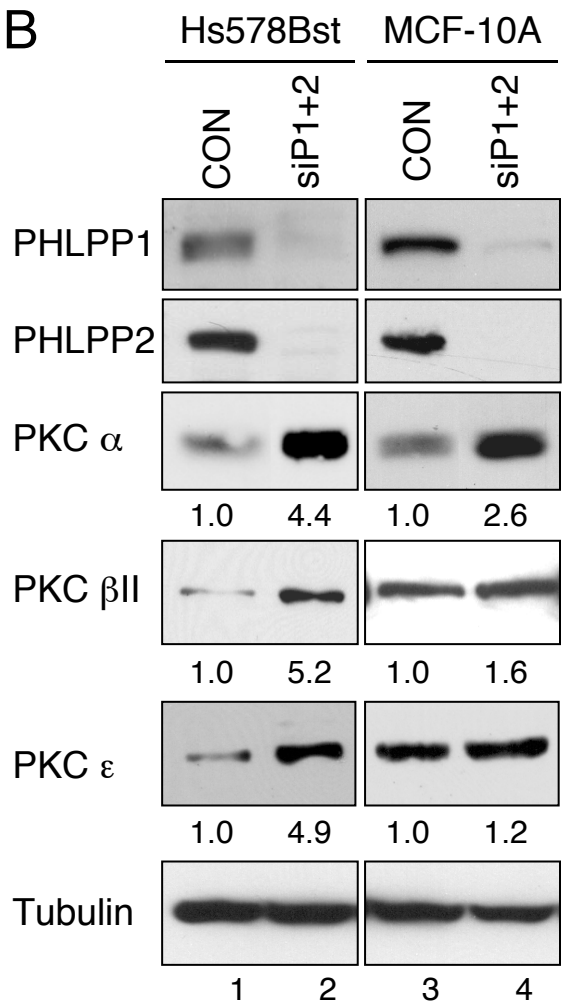

Figure 8