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# PHLPP: A Phosphatase that Directly Dephosphorylates Akt, Promotes Apoptosis, and Suppresses Tumor Growth

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## Summary

**Akt/protein kinase B critically regulates the balance between cell survival and apoptosis. Phosphorylation of Akt at two key sites, the activation loop and the hydrophobic motif, activates the kinase and promotes cell survival. The mechanism of dephosphorylation and signal termination is unknown. Here, we identify a protein phosphatase, *PH* domain *l*eucine-rich repeat *p*rotein *p*hosphatase (PHLPP), that specifically dephosphorylates the hydrophobic motif of Akt (Ser473 in Akt1), triggering apoptosis and suppressing tumor growth. The effects of PHLPP on apoptosis are prevented in cells expressing an S473D construct of Akt, revealing that the hydrophobic motif is the primary cellular target of PHLPP. PHLPP levels are markedly reduced in several colon cancer and glioblastoma cell lines that have elevated Akt phosphorylation. Reintroduction of PHLPP into a glioblastoma cell line causes a dramatic suppression of tumor growth. These data are consistent with PHLPP terminating Akt signaling by directly dephosphorylating and inactivating Akt.**

## Introduction

The balance between cell survival and apoptosis critically controls normal cell growth. Akt/protein kinase B regulates this balance through a phosphorylation cascade that primarily alters the function of transcription factors that regulate pro- and antiapoptotic genes (Datta et al., 1999; Franke et al., 1997). Misregulation of the Akt signaling pathway is a key cause of cancer (Black, 2000; Lawlor and Alessi, 2001; Nicholson and Anderson, 2002; Vivanco and Sawyers, 2002). Akt is activated by growth factors and other stimuli that cause generation of the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PtdIns P3) through activation of phosphoinositide-3-kinase (PI3 kinase). This lipid product recruits Akt to the membrane by engaging its PH domain, an event that triggers phosphorylation of Akt. After phosphorylation, Akt is locked in an active conformation and is released into the cytosol and nucleus where it phosphorylates substrates such as Forkhead transcription factors, BAD, and GSK. The tumor suppressor PTEN is a lipid phosphatase that controls Akt function by dephosphorylating PtdIns P3, thus removing the activating signal and ultimately preventing Akt phosphorylation and activation (Maehama and Dixon, 1998; Wishart and Dixon, 2002). How Akt signaling is terminated once it has been initiated is unknown. Specifically, the dephosphorylation mechanism to directly inactivate Akt has yet to be elucidated.

Akt is activated by sequential phosphorylation steps at two sites conserved within the AGC kinase family (Newton, 2003). First, the upstream kinase PDK-1 phosphorylates a segment at the entrance to the active site termed the activation loop (Alessi et al., 1998; Stephens et al., 1998). In Akt1, the residue phosphorylated is Thr308. The phosphorylation by PDK-1 triggers the phosphorylation of a site at the carboxyl terminus referred to as the hydrophobic phosphorylation motif and corresponds to Ser473 in Akt1. The mechanism of phosphorylation at this carboxyl-terminal site has been proposed to occur by autophosphorylation (Behn-Krappa and Newton, 1999; Toker and Newton, 2000). However, it has been debated whether a unique, and as yet unidentified, kinase, tentatively referred to as PDK-2, controls this site (Leslie et al., 2001). In the case of Akt's close cousin, protein kinase C, mechanistic studies have revealed that the phosphorylation reactions of the activation loop site and hydrophobic site are tightly coupled (Gao et al., 2001). However, the actual phosphorylation state of the corresponding sites on Akt is often uncoupled in cells. For example, in Akt, Thr308 has been reported to be dephosphorylated much more rapidly than Ser473 after decay of the insulin signal (Yamada et al., 2001). Similarly, staurosporine treatment results in accumulation of a species of Akt that has phosphate on Ser473, but not on Thr308 (Hill et al., 2001). Assuming tight coupling of the phosphorylation steps in Akt, we hypothesized that the dephosphorylation of the two sites is uncoupled. Thus, we set out to determine if there exists a phosphatase (rather than kinase) that regulates the phosphorylation state of the hydrophobic motif. Based on the serum-sensitivity of the phosphorylation state of Akt, we hypothesized that such a phosphatase might contain a PH domain.

Here, we report on the discovery of a protein phosphatase, identified from a database search of the human genome for a phosphatase linked to a PH domain, that specifically dephosphorylates the hydrophobic motif of Akt. Biochemical and cellular studies are consistent with PHLPP terminating Akt signaling by direct dephosphorylation of the hydrophobic motif, providing a distinct mechanism for signal termination from that mediated by the lipid phosphatase PTEN.

## Results

A search of the database for a sequence that contained a PH domain coupled to a phosphatase domain revealed a rat sequence that fulfilled these criteria. This gene product had been named SCOP (suprachiasmatic nucleus circadian oscillatory protein) to reflect the observation that mRNA levels in the suprachiasmatic nucleus were found to oscillate in a circadian rhythm-dependent manner (Shimizu et al., 1999). The protein product of the message was not functionally charac-

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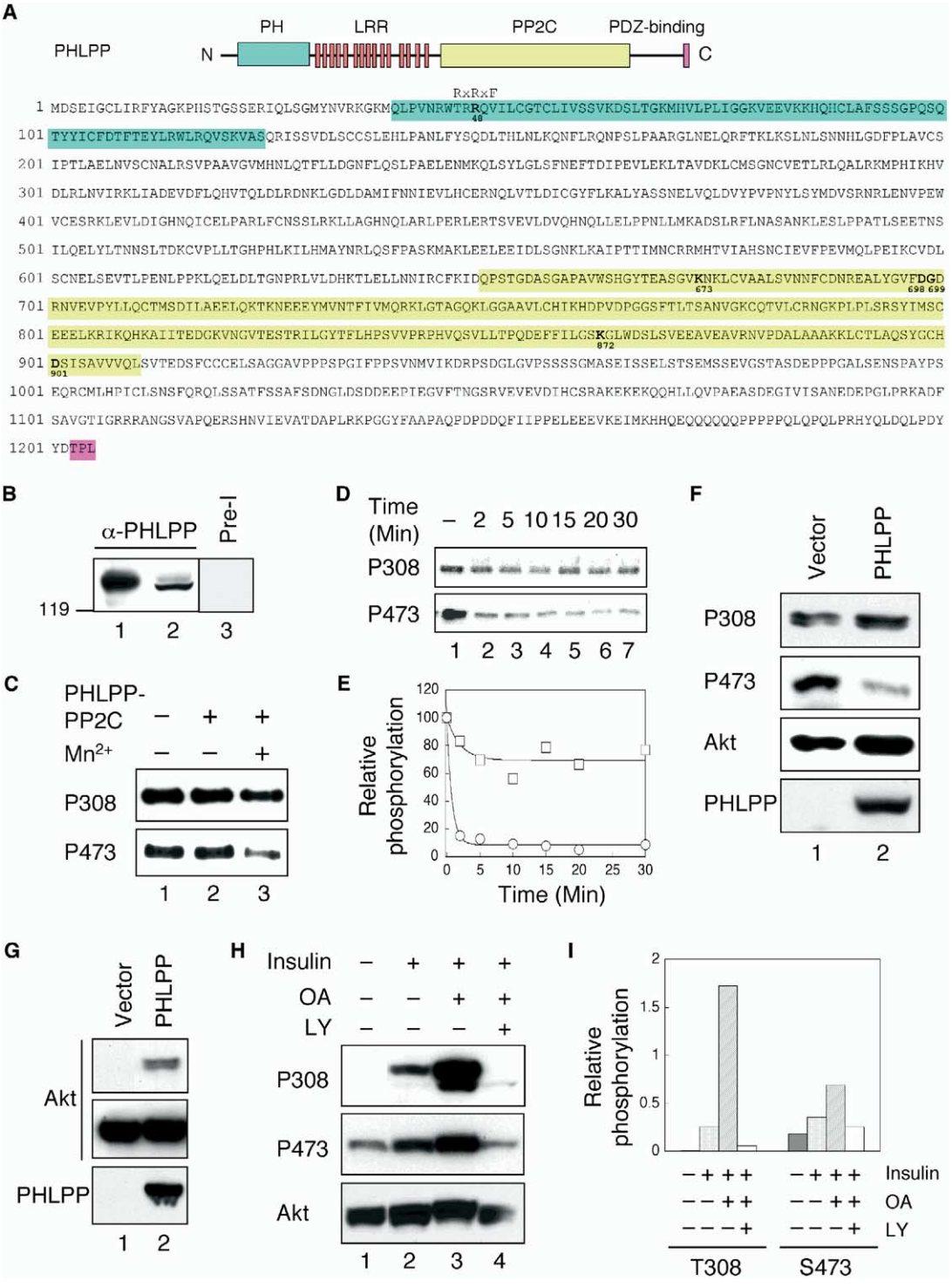


Figure 1. PHLPP, a Protein Phosphatase, Dephosphorylates Akt In Vitro and In Vivo

(A) Schematic representation and amino acid sequence of human PHLPP, a 1205 residue protein predicted by Pfam (http://pfam.wustl.edu) to contain an amino-terminal PH domain (cyan), a leucine-rich repeat region (LRR, red), a PP2C-like catalytic core (yellow), and a PDZ binding motif (fuchsia). The NCBI accession number for PHLPP is NM_194449. The amino acid residues corresponding to the PH domain, PP2C domain, and PDZ binding motif are boxed in cyan, yellow, and fuchsia, respectively. The consensus residues dictating phosphoinositide binding are indicated above the PH domain (Ferguson et al., 2000); of these, only Arg48 is conserved in PHLPP. The residues delineated for the PP2C domain are based on the structure determined for PP2Cα (Das et al., 1996); key residues identified for phosphate binding (Arg33) and metal binding (Asp60, Gly699, Asp239, and Asp282) in human PP2Cα are indicated in bold and correspond to Lys673, Asp698, Gly699, Lys872, and Asp901 in PHLPP, respectively (Jackson et al., 2003).

terized. We cloned the human homolog by combining the cDNA fragments described in the Experimental Procedures. The nucleotide sequence of the cloned product was predicted to encode a 1205 amino acid protein with a PH domain, a leucine-rich repeat region, a PP2C domain, and a PDZ binding motif (Figure 1A; domains and conserved residues within these domains are indicated). We named the protein to reflect its domain structure: PHLPP for *PH* domain *l*eucine-rich repeat *p*rotein *p*hosphatase (pronounced "flip"). Based on the tissue distribution of available PHLPP ESTs and microarray analysis results found at http://genome-www.stanford.edu, the mRNA of PHLPP is ubiquitously expressed, with highest levels in brain.

To identify the expression of endogenous PHLPP protein, a specific antibody was raised against a C-terminal fusion protein of PHLPP. This antibody detected a ~140 kDa protein in 293T cells transfected with the cDNA for HA-tagged PHLPP. In untransfected cells, it detected a doublet of apparent MW 135 (major band) and 140 kDa (minor band) (Figure 1B, lanes 1 and 2).

We first addressed whether the PP2C domain of PHLPP was catalytically active. The PP2C domain was expressed in bacteria as a GST fusion protein, purified, and incubated with pure baculovirus-expressed Akt, which is phosphorylated on Thr308 and Ser473 (Toker and Newton, 2000). The Western blot in Figure 1C shows that the PHLPP PP2C domain dephosphorylated Ser473 and, less effectively, Thr308 as probed with phospho-specific antibodies to each site. This phosphatase activity depended on $Mn^{2+}$, consistent with previous reports that the activity of PP2C phosphatases is metal dependent (Fjeld and Denu, 1999). Under the conditions of the dephosphorylation assays (5 mM $Mn^{2+}$, 0.3 μM Akt, and 5 nM PP2C), the time course in Figures 1D and 1E revealed a half time of 0.53 ± 0.08 min for dephosphorylation of Ser473, corresponding to a catalytic rate of 1 dephosphorylation reaction per sec. Thr308 was dephosphorylated at a third this rate. For comparison, the $k_{cat}$ of recombinant PP2Cα toward saturating synthetic substrate (20 mM *p*NPP) in the presence of saturating $Mn^{2+}$ has been reported to be 1 $s^{-1}$ (Fjeld and Denu, 1999). Thus, even at concentrations of substrate that are likely well below the Km, PHLPP has a catalytic rate equivalent to that reported for PP2Cα.

We next asked whether full-length PHLPP could dephosphorylate Akt in cells. 293T cells were cotransfected with Akt and empty vector or HA-tagged, full-length PHLPP and maintained in serum to activate Akt. Western blot analysis of cell lysates revealed that the phosphorylation state of Akt at Ser473 was markedly reduced in cells transfected with HA-PHLPP (Figure 1F, lane 2) compared with cells transfected with vector alone (Figure 1F, lane 1). Given a transfection efficiency of ~70%–80%, Ser473 phosphorylation was essentially abolished in cells overexpressing PHLPP. In striking contrast, coexpression of PHLPP had no significant effect on the phosphorylation of Thr308. Total Akt levels were similar in control cells and those expressing recombinant PHLPP. These data reveal that PHLPP selectively regulates the hydrophobic motif, but not the activation loop site, of Akt in cells.

To explore whether PHLPP interacts with Akt in cells, we asked whether the proteins coimmunoprecipitate. 293T cells were transfected with HA-tagged, full-length PHLPP and Flag-tagged Akt. Figure 1G shows that Akt was present in immunoprecipitates of full-length PHLPP (lane 2), consistent with the two proteins forming a complex in cells.

The activity of PP2C family members is not sensitive to okadaic acid (OA) (Cohen and Cohen, 1989). Thus, if PHLPP controls the phosphorylation state of Ser473, then phosphorylation of this site should be relatively insensitive to OA. Figure 1H shows that the insulin-

(B) Detergent-solubilized lysates from 293T cells transfected with HA-PHLPP (lane 1) or vector alone (lanes 2 and 3) were analyzed by SDS-PAGE and immunoblotting. Overexpressed HA-PHLPP or endogenous PHLPP was detected by an anti-PHLPP antibody (lanes 1 and 2, respectively). No labeling was observed by using a preimmune anti-serum (lane 3).

(C) Dephosphorylation of Akt by purified PP2C domain of PHLPP in vitro. Pure His-Akt was incubated with GST-tagged PHLPP-PP2C in the absence or presence of 5 mM $MnCl_2$ (lanes 2 and 3, respectively). For a negative control, PHLPP-PP2C was omitted from the reaction mixture (lane 1). The phosphorylation of Akt at Thr308 and Ser473 was detected by P308 and P473 antibodies, respectively.

(D) Western blots showing the time course of GST-tagged PHLPP-PP2C mediated dephosphorylation of Akt. Pure His-Akt was incubated with GST-PP2C for 2, 5, 10, 15, 20, and 30 min (lanes 2–7, respectively). As a negative control, PHLPP-PP2C was omitted from the reaction mixture (lane 1). The phosphorylation of Akt at Thr308 and Ser473 was detected by using P308 and P660 (a phosphospecific antibody for the hydrophobic phosphorylation motif of PKC βII [Ser660] that recognizes phosphorylated S473 of Akt with greater phosphospecificity than P473 [Toker and Newton, 2000]) antibodies, respectively.

(E) Quantitative analysis of Western blots presented in (D). ECL signals of P308 and P473 antibodies were quantified by a CCD camera using a GeneGnome bioimaging system. The relative phosphorylation of Akt at Thr308 (open square) and Ser473 (open circle) was normalizing to the zero time point. The curve represents a single exponential fit of the data points.

(F) Western blots showing dephosphorylation of overexpressed Akt in intact cells by PHLPP. 293T cells were cotransfected with HA-tagged Akt and either vector (lane 1) or HA-PHLPP (lane 2). The phosphorylation of Akt was detected by using P308 and P473 antibodies. The total HA-Akt was detected by an anti-Akt antibody. The expression of HA-PHLPP was detected by an anti-HA antibody.

(G) PHLPP interacts with Akt in vivo. The presence of Akt in the immunoprecipitate and lysate (10% of total input) was detected by using the anti-Akt antibody (top and middle, respectively). The presence of HA-PHLPP in the immunoprecipitates was detected by using the anti-HA antibody (bottom).

(H) PHLPP-mediated dephosphorylation of Akt at Ser473 is okadaic acid (OA) insensitive. 293T cells transfected with HA-PHLPP were serum starved overnight. The cells were then left untreated (lane 1), treated with insulin (100 nM) for 30 min (lane 2), treated with insulin for 30 min followed by OA (1 μM) for 15 min (lane 3), or treated with insulin for 30 min followed by OA for 15 min followed by LY294002 (LY, 30 μM) for 20 min (lane 4). Western blots showing phosphorylation of endogenous Akt as detected by P308 and P473 antibodies. Total Akt was detected by using the anti-Akt antibody.

(I) Quantitative analysis of data from the Western blots shown in (F). Relative phosphorylation of Akt at Thr308 and Ser473 was determined by normalizing ECL signals of P308 and P473 signal intensities to those of the anti-Akt antibody.

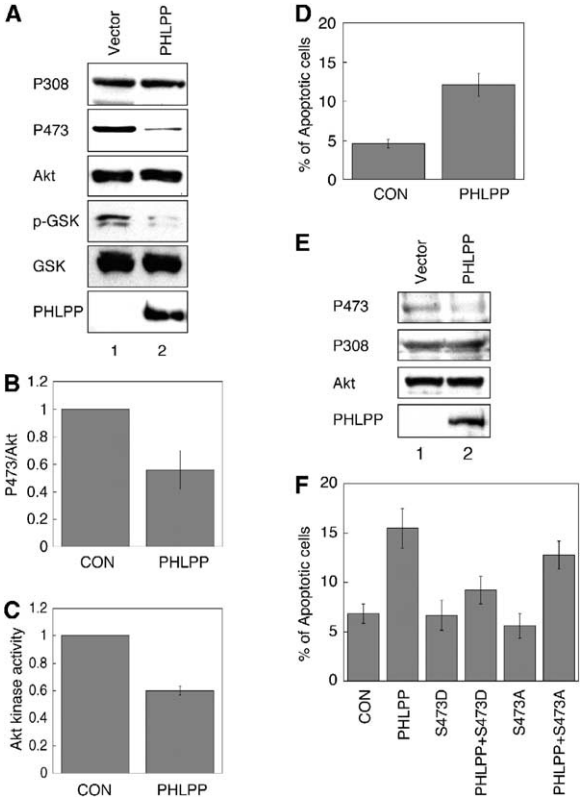


Figure 2. Overexpression of HA-PHLPP in Cancer Cells Induces Dephosphorylation of Endogenous Akt at Ser473 and Triggers Apoptosis

(A) Western blots showing phosphorylation of endogenous Akt and Akt substrate GSK from H157 cells transfected with vector (lane 1) or HA-PHLPP (lane 2). The phosphorylation of endogenous Akt was detected by using P308 and P473 antibodies. The total Akt was detected by using the anti-Akt antibody. The phosphorylation of GSK$\alpha/\beta$ at the Akt site (Ser21 in GSK$\alpha$ and Ser9 in GSK$\beta$) was detected by using a phospho-specific antibody (p-GSK), whereas the total GSK$\alpha/\beta$ was detected by using an anti-GSK antibody (GSK). The expression of HA-PHLPP was detected by using the anti-HA antibody.

(B) Relative phosphorylation of Akt at Ser473 (P473/Akt) was determined by normalizing ECL signals of P473 antibody to those of the anti-Akt antibody, and the numbers for the vector-transfected control (CON) cells were normalized to 1. Data represent mean ± SEM (n = 3).

(C) Graph showing Akt kinase activity was reduced in HA-PHLPP-expressing H157 cells. In vitro kinase assays were performed with endogenous Akt immunoprecipitated from the CON or HA-PHLPP transfected cells. The Akt activity in the CON cells was set to 1. Data represent mean ± SEM (n = 3).

(D) Effect of overexpressing PHLPP on cellular apoptosis. H157 cells were cotransfected with plasmids encoding GFP and either vector (CON) or HA-PHLPP (PHLPP). Transfected apoptotic cells were sorted based on GFP expression and assayed to determine sub-2N DNA content. The bar graph presents the mean ± SEM (n = 4).

(E) Western blots showing phosphorylation of endogenous Akt from MDA-MB-231 cells transfected with vector (lane 1) or HA-PHLPP (lane 2) as probed with P308 and P473 antibodies. The total Akt was detected by using the anti-Akt antibody. The expression of HA-PHLPP was detected by using the anti-PHLPP antibody.

(F) Graph showing the rescue of PHLPP-induced apoptosis by coexpression of an Akt/S473D mutant construct. MDA-MB-231 cells were transiently transfected with vector (CON), HA-PHLPP (PHLPP), HA-Akt/S473D (S473D), HA-PHLPP plus HA-Akt/S473D (PHLPP+S473D), HA-Akt/S473A (S473A), or HA-PHLPP plus HA-Akt/S473A (PHLPP+S473A). The apoptotic cells were determined based on the sub-2N DNA content. The bar graph presents the mean ± SEM (n = 3).

dependent phosphorylation of Thr308 displayed marked sensitivity to OA. Specifically, Thr308 was phosphorylated to a 7-fold higher level in cells treated with OA after insulin treatment compared to cells not exposed to the phosphatase inhibitor (Figure 1I). In marked contrast, the phosphorylation of Ser473 displayed only modest sensitivity: less than a 2-fold effect of OA on phosphorylation of this site was observed (Figure 1I). The relative insensitivity of the phosphorylation state of Ser473 to OA is consistent with PHLPP being the key protein phosphatase for this site.

We next explored the effect of PHLPP-catalyzed dephosphorylation of Akt on cellular apoptosis. Full activation of Akt requires phosphorylation of both Thr308 and Ser473. Enzyme phosphorylated on both residues has a 20-fold higher specific activity than enzyme phosphorylated only on Thr308 (Alessi et al., 1996). However, phosphorylation of Ser473 may be the critical regulator of activity. because selective dephosphorylation of Thr308 does not significantly affect the activity of Akt (Yamada et al., 2001). This is similar to protein kinase C, where phosphate at the PDK-1 site becomes dispensable after phosphorylation at the carboxyl-terminal autophosphorylation sites (Keranen et al., 1995). To address whether dephosphorylation of Ser473 could terminate Akt signaling and affect cell survival, we monitored cell apoptosis as a function of overexpressing PHLPP (Figure 2). H157 cells are derived from non-small cell lung cancers and have elevated Akt phosphorylation and low apoptosis (Brognard et al., 2001). Figure 2A shows that Akt is phosphorylated on both Thr308 and Ser473 in these cells (lane 1). Expression of PHLPP reduced phosphorylation of Ser473 to ~50% of the level observed in control cells; PHLPP expression had no detectable effect on the phosphorylation of Thr308 (Figures 2A and 2B, lane 2). This decrease in Akt phosphorylation on Ser473 correlated directly with (1) decreased phosphorylation of an Akt substrate, GSK (Figure 2A), and (2) reduction of Akt activity in cells (Figure 2C). These data reveal that phosphorylation of Ser473 is essential for maintaining full Akt activity. The effect of overexpressing PHLPP on the apoptotic rate of H157 cells was assayed by using flow cytometry. Apoptotic cells were identified by sorting propidium iodide-stained cells based on DNA content with the fraction of cells with sub-2N DNA considered apoptotic. Coexpression of PHLPP caused a 2.6-fold increase in the number of apoptotic cells from 4.6% ± 0.5% to 12% ± 2% (Figure 2D). Transfection efficiency in these cells was 50%–60%, suggesting that Ser473 phosphorylation and Akt activity were abolished in cells overexpressing PHLPP. Thus, dephosphorylation of the hydrophobic site is accompanied by decreased Akt activity and increased apoptosis.

If the ability of PHLPP to increase cellular apoptosis results from dephosphorylation of Ser473, we reasoned that cells transfected with the phospho-mimetic S473D

construct of Akt should be resistant to PHLPP. Thus, we set out to explore the apoptotic level of cells transfected with PHLPP alone or PHLPP and the S473D construct of Akt. For these experiments, we focused on the breast cancer cell line MDA-MB-231 because, unlike the H157 cells, they tolerated well transfection of the hyperactive Akt S473D construct; these cells have elevated phosphorylation of Ser473, which is reduced after transfection of PHLPP (Figure 2E). Figure 2F shows that expression of PHLPP in these cells increased the number of apoptotic cells 2.3-fold, from 7% ± 1% to 16% ± 2%, as observed for H157 cells. Cotransfection of the S473D construct of Akt with PHLPP inhibited this increase in apoptotic cells by ~80%: the number of apoptotic cells was 9% ± 1% in the presence of S473D. Expression of the S473D construct alone had no effect on the number of apoptotic cells. Cotransfection of the construct S473A and PHLPP also inhibited apoptosis, but the effect was markedly reduced (30% reduction in apoptosis) compared to the S473D construct. It is possible that the S473A had a slight effect on apoptosis by competitively inhibiting PHLPP; consistent with this, the S473A construct alone reduced the number of apoptotic cells by ~20%. Importantly, the S473D construct effectively rescued the PHLPP-dependent increase in apoptosis, revealing that the major mechanism by which PHLPP increases apoptosis is by dephosphorylation of Ser473 on Akt. Comparable effects were observed in H157 cells (data not shown).

To explore the role of potential regulatory regions in PHLPP, mutant constructs were generated in which the PH domain (ΔPH) or carboxyl-terminal 3 residues, encoding a potential PDZ binding motif (ΔC), were deleted (Figure 3A). The Western blot in Figure 3B and corresponding graph quantifying the data (Figure 3C) show that wild-type (wt) PHLPP reduced the steady-state phosphorylation of Akt by ~50% in H157 cells growing in serum. Deletion of the PH domain resulted in slightly increased phosphatase activity. In contrast, deletion of the PDZ binding motif suppressed the ability of PHLPP to dephosphorylate Akt in cells. All PHLPP constructs were expressed to comparable levels in cells and had no effect of the level of endogenous Akt. Analysis of the number of apoptotic cells revealed that dephosphorylation of Ser473 correlated with apoptosis. Specifically, PHLPP caused a 3-fold increase in apoptotic cells, the PH domain-deleted construct caused a 3.5-fold increase, and the construct deleted in the PDZ binding motif suppressed the ability of PHLPP to increase apoptosis (Figure 3D). Note that deletion of the PDZ binding motif does not impair the intrinsic phosphatase activity of PHLPP as assessed by in vitro dephosphorylation assays using immunoprecipitated construct (data not shown). This suggests that targeting of PHLPP by its PDZ binding motif is required for its biological function.

We next assessed the effects of depleting cells of endogenous PHLPP by using siRNAs to knockdown endogenous PHLPP in H157 cells. Figure 4A shows that two siRNA sequences (Si-2 and Si-3) directed against different regions of the PHLPP sequence effectively reduced endogenous PHLPP in 293T cells. (We used 293T cells to test for the best siRNA sequences, because endogenous PHLPP is readily detectable in

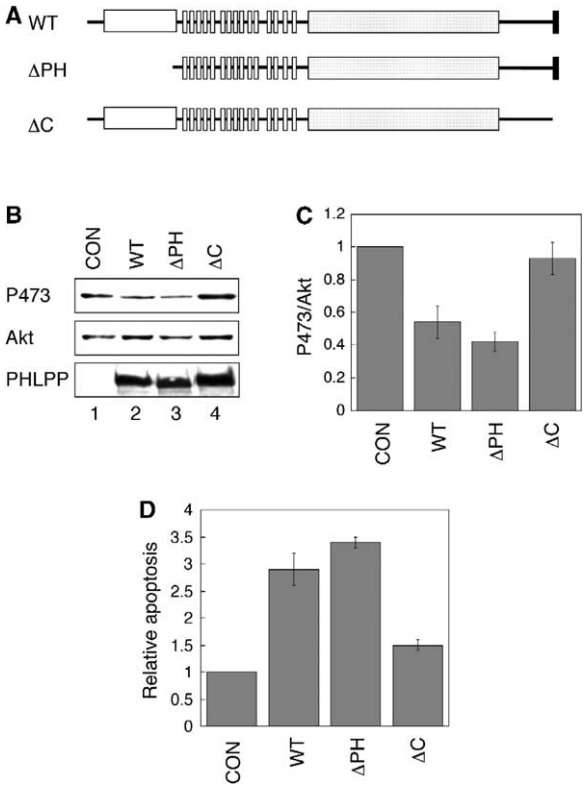


Figure 3. The Function of the PH Domain and the PDZ Binding Motif in PHLPP

(A) Schematic representation of the wild-type (wt) and mutant PHLPP constructs.

(B) Western blots of H157 cell lysates transfected with vector (CON), HA-PHLPP, HA-ΔPH, or HA-ΔC (lanes 1–4, respectively). Phosphorylation of endogenous Akt at Ser473 was detected by using P473 antibody (top), whereas the total Akt was detected by using the anti-Akt antibody (middle). The expression of PHLPP was detected by using the anti-PHLPP antibody (bottom).

(C) Relative phosphorylation of Akt at Ser473 (P473/Akt) was determined by normalizing ECL signals of P473 antibody to those of the anti-Akt antibody, and the numbers for the vector-transfected cells (CON) were normalized to 1. Data represent mean ± SEM (n = 3).

(D) H157 cells transfected with the constructs described in (E) were analyzed with flow cytometry to determine the number of apoptotic cells. A GFP expression construct was cotransfected into the cells to allow sorting for transfected cells. The number of apoptotic cells in vector-transfected cells was normalized to 1 (CON). Data represent the mean ± SEM (n = 3).

these cells. This contrasts to all cancer cell lines we tested in which PHLPP levels are greatly reduced and difficult to quantify by Western blot analysis; for these cells, quantitative RT-PCR was used to measure the ability of specific siRNA sequences to knockdown PHLPP and comparable results were obtained). Si-2 and Si-3 were used to reduce endogenous PHLPP expression in H157 cells. Figures 4B and 4C show that transfection of either siRNA against PHLPP resulted in an increase in Akt phosphorylation at Ser473, both in cells grown in low-serum media (lanes 1–3) and also in cells treated with LY294002 to inhibit PI3 kinase (lanes 4–6). Because H157 cells do not express functional PTEN (Brognard et al., 2001), Akt phosphorylation is elevated basally and

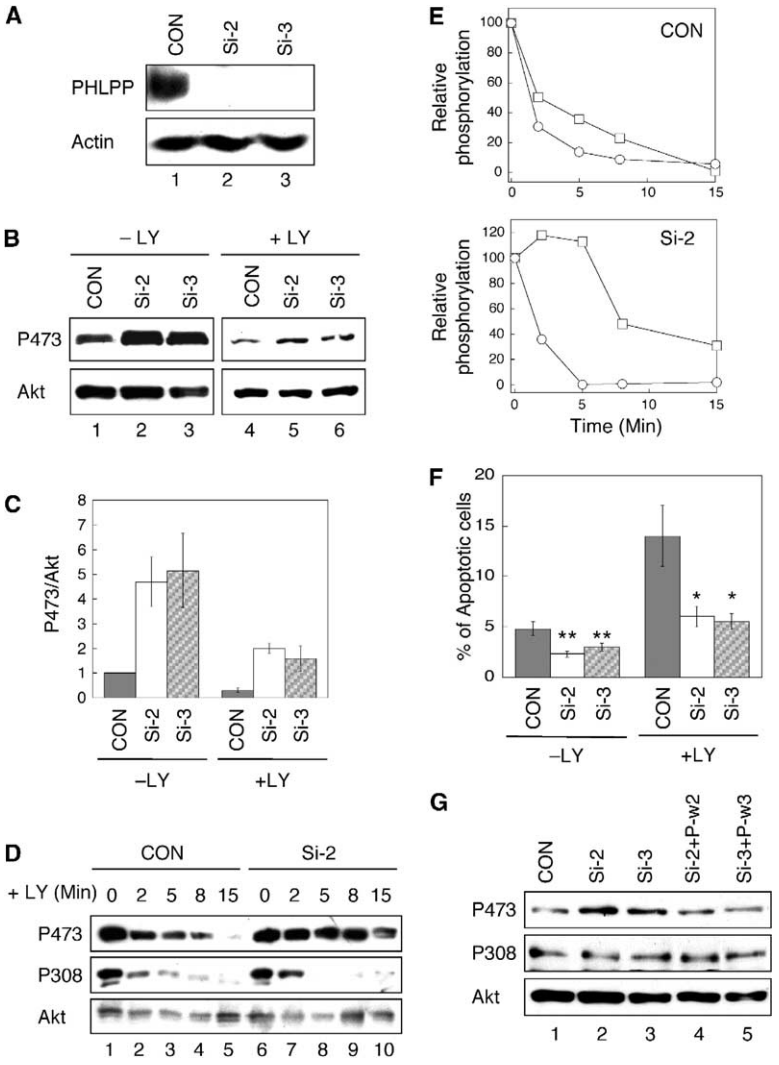


Figure 4. Downregulation of Endogenous PHLPP Prevents Apoptosis of H157 Cells

(A) 293T cells were transfected with CON siRNA (lane 1), or two siRNAs against different regions of the PHLPP gene (lanes 2 and 3, Si-2 and Si-3, respectively). Expression of endogenous PHLPP was detected by using the anti-PHLPP antibody (top). Equal total protein levels were verified by probing the same blot with an anti-actin antibody (bottom).

(B) Western blots from siRNA-transfected H157 cells demonstrate increased phosphorylation of Akt at Ser473. H157 cells were transfected with control (scrambled) siRNA (lanes 1 and 4), Si-2 (lanes 2 and 5) and Si-3 (lanes 3 and 6). 24 hr posttransfection, the cells were placed in 0.1% FBS for 48 hr in the absence (lanes 1–3) or presence of LY294002 (20 μM, lanes 4–6). The phosphorylation of Akt at Ser473 was detected by using P473 (top); total Akt was detected by using the anti-Akt antibody (bottom).

(C) Graph showing quantified P473 phosphorylation data from three independent experiments as shown in (B). Relative phosphorylation of Akt at Ser473 (P473/Akt) was determined by normalizing ECL signals of P473 antibody to those of the anti-Akt antibody, and the vector transfected cells under control conditions (CON) were set to 1. Data represent mean ± SEM (n = 3).

(D) Western blots showing the time course of LY294002-induced dephosphorylation of endogenous Akt. H157 cells expressing CON siRNA (lanes 1–5) or Si-2 (lanes 6–10) were treated with LY294002 (15 μM) for 0, 2, 5, 8, and 15 min. The phosphorylation of Akt at Thr308 and Ser473 was detected by using P308 and P473 antibodies, respectively. The total Akt protein was detected by using the anti-Akt antibody.

(E) Quantitative analysis of Western blots shown in (D). The relative phosphorylation of Akt at Thr308 (open circle) and Ser473 (open square) was quantified by normalizing ECL signals of P308 and P473 antibodies to those of the anti-Akt antibody; the amount of Akt phosphorylation at the 0 time point was normalized to 100%. Data for cells transfected with CON or Si-2 are shown on the top or the bottom, respectively.

(F) Quantified results of apoptosis analysis of siRNA-transfected H157 cells. H157 cells were transfected with CON, Si-2, and Si-3. Apoptosis was quantified by using flow cytometry. The bar graph summarizes four independent experiments showing the mean ± SEM. Student's t tests were performed to compare PHLPP siRNA-treated cells with the control cells, and p values are <0.01 (**) and <0.05 (*) for cells treated with LY and without LY, respectively.

(G) Western blots showing PHLPP siRNA-induced increase of Akt phosphorylation at Ser473 was blocked by coexpression of PHLPP constructs carrying silent mutations in the siRNA-targeting sequence (PHLPP-w2 and -w3). H157 cells were transfected with control siRNA, Si-2, Si-3, Si-2+PHLPP-w2 (P-w2), and Si-3+PHLPP-w3 (P-w3) (lanes 1–5, respectively). The phosphorylation of Akt at Ser473 was detected by using P473 (top) and P308 (middle); total Akt was detected by using the anti-Akt antibody (bottom).

reduced 2- to 3-fold by inhibition of PI3 kinase (compare lanes 1 and 4). Analysis of the rate of LY294002-induced dephosphorylation of Akt revealed that Ser473 and Thr308 were dephosphorylated with a half time of ~2 min under the conditions examined in control cells (Figures 4D and 4E). Knockdown of PHLPP by siRNA had no significant effect on the rate of dephosphorylation of Thr308, but reduced the rate of dephosphorylation of Ser473 ~5-fold. These data reveal that PHLPP specifically controls the phosphorylation state of Ser473.

Apoptosis assays were performed to examine the functional effect of expressing siRNAs against PHLPP. Figure 4F shows that control cells grown in low serum had low numbers of apoptotic cells (4.8% ± 0.7%). Knockdown of endogenous PHLPP resulted in an even lower number of apoptotic cells (2.3% ± 0.3% and 3.0% ± 0.4% for Si-2 and Si-3 siRNA, p value < 0.01). This correlated with the increased phosphorylation of Akt presented in Figure 4B. Inhibition of PI3 kinase tripled the number of apoptotic cells in control samples to ~14% ± 3%, correlating with the decrease in Akt phosphorylation presented in Figure 4B. Knockdown of PHLPP prevented this increase: 6% ± 1% and 5.5% ± 0.8% of siRNA-treated cells were apoptotic for the Si-2 and Si-3 (p value < 0.05), respectively. In control

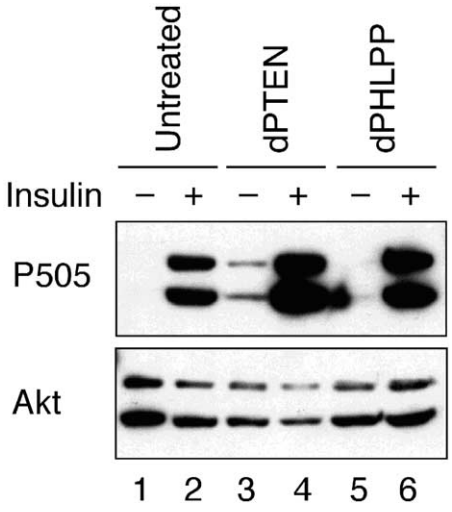


Figure 5. Downregulation of a PHLPP Homolog, dPHLPP, in *Drosophila* Cells Results in Elevated Phosphorylation of Akt at the Hydrophobic Motif

*Drosophila* S2 cells were treated with specific dsRNAs against dPHLPP (lanes 5 and 6) or dPTEN (lanes 3 and 4). Untreated S2 cells were used as negative controls (lanes 1 and 2). The cells were serum starved for 2 hr and then treated with insulin (300 nM) for 5 min. The cell lysates were analyzed for Akt phosphorylation at the hydrophobic motif (Ser505 in dAkt) by using the P505 phospho-specific antibody (top). The total Akt in the lysates was detected by using the anti-Akt antibody (bottom).

experiments, expression constructs of the full-length PHLPP were created to introduce silent mutations in the siRNA-targeting sequences. Figure 4G shows that coexpression of these constructs (lanes 4 and 5) abolished the effects of both Si-2 and Si-3 siRNAs (lanes 2 and 3) to increase Ser473 phosphorylation relative to untransfected cells (lane 1). Note that phosphorylation of Thr308 was not affected by siRNA treatment. These data reveal that endogenous PHLPP catalyzes the dephosphorylation of Akt at Ser473 in vivo and that this dephosphorylation leads to apoptosis.

We asked the question whether PHLPP-mediated downregulation of Akt signaling is also conserved in lower organisms such as *Drosophila*, because this survival pathway is well conserved from fly to human. By searching the *Drosophila* gene database, a PHLPP homolog was identified that had a similar domain composition as mammalian PHLPP but lacked the N-terminal PH domain. We named the *Drosophila* PHLPP homolog dPHLPP. To test whether dPHLPP serves as the hydrophobic motif phosphatase for *Drosophila* Akt (dAkt) as well, we used double-stranded RNA (dsRNA) to knockdown endogenous dPHLPP in fly S2 cells. As a control, dsRNA against *Drosophila* PTEN (dPTEN) was used to knockdown endogenous dPTEN (Clemens et al., 2000). Figure 5 shows that knocking down dPHLPP resulted in a robust increase in Akt phosphorylation at the hydrophobic motif (Ser505 in dAkt), similar to that observed by knocking down dPTEN. Thus, the PHLPP-mediated negative regulation of Akt signaling is conserved in *Drosophila*, similar to the negative regulation mediated by the lipid phosphatase PTEN.

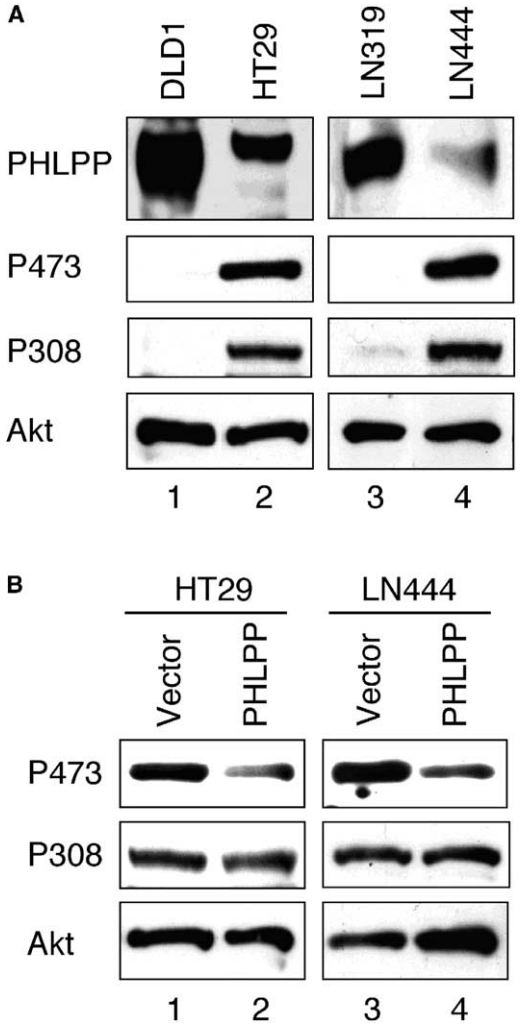


Figure 6. Reduced Expression Levels of PHLPP in Cancer Cell Lines Correlate with Elevated Akt Phosphorylation

(A) Western blot analysis of two colon cancer cell lines, DLD1 and HT29, and two glioblastoma cell lines, LN319 and LN444. Detergent-soluble cell lysates were probed with antibodies to PHLPP (top), phosphorylated Ser473 and Thr308 (P473 and P308, respectively), or total Akt (bottom).

(B) Cancer cell lines HT29 (lanes 1 and 2) and LN444 (lanes 3 and 4) were transfected with vector (lanes 1 and 3) or HA- PHLPP (lanes 2 and 4). Detergent-soluble cell lysates were analyzed with antibodies to phosphorylated Thr308 (P308), Ser473 (P473), or total Akt (Akt).

PHLPP is located on chromosome 18q21.33, a locus that represents one of the most highly lost regions in colon cancer (Goel et al., 2003; Jen et al., 1994). Thus, we screened five cell lines derived from colon tumors for PHLPP expression and Akt phosphorylation. All cell lines had wt PTEN. The Western blot in Figure 6A shows that cell line HT29 (lane 2) had markedly reduced levels of PHLPP compared to cell line DLD1 (lane 1). Both cell lines had comparable amounts of total Akt; however, the phosphorylation levels of Ser473 and Thr308 were greatly elevated in HT29 cells relative to DLD1 cells. PTEN levels were comparable for both cell

lines (data not shown). The low levels of PHLPP and high level of Akt phosphorylation in the cell line HT29 were representative of four out of the five cell lines examined (data not shown). Given the abundance of PHLPP in brain, we also screened six glioblastoma cell lines for PHLPP levels, and results from two cell lines are shown in Figure 6A. LN444 had barely detectable PHLPP relative to LN319 (lanes 3 and 4). Both cell lines have comparable levels of Akt, but the phosphorylation of Ser473 and Thr308 was dramatically increased in LN444 cells. Both cell lines express nonfunctional PTEN as reported previously (Furnari et al., 1997). Transfection of wt PHLPP into HT29 and LN444 cells resulted in a decrease of Akt phosphorylation at Ser473 compared to cells transfected with vector alone, with no effect on phosphorylation of Thr308 (Figure 6B). Given that the transfection efficiency was 30%–50%, these data reveal that phosphorylation was essentially abolished in the cells expressing recombinant PHLPP. These data show that for cell lines from two different cancers, colon and glioblastoma, reduced levels of PHLPP correlate with high levels of phosphorylation on Ser473 of Akt. Reintroduction of PHLPP into the cells expressing low levels of the phosphatase leads to dephosphorylation of Akt.

We next addressed whether introduction of PHLPP into tumor cells affects cell growth and tumor formation in animals. The human glioblastoma cell line LN229 is highly tumorigenic (Furnari et al., 1997; Ishii et al., 1999). However, overexpression of PTEN in these cells has been shown to have no inhibitory effect on cell growth and tumorigenicity because wt PTEN is expressed (Furnari et al., 1997). Endogenous PHLPP was not detected in LN229 cells by using our anti-PHLPP antibody (data not shown), suggesting a possible defect in this protein in these cells. To assess this, we tested the effect of reintroducing the wt PHLPP on cell growth and tumorigenicity in these cells. Figure 7A shows that the growth rate of LN229 cells stably transfected with HA-PHLPP (PHLPP) was reduced by ~50% compared to that of vector-transfected control cells (CON). Phosphorylation of Akt at Ser473 and Thr308 was markedly decreased in cells stably expressing HA-PHLPP (Figure 7B, lane 2). Note that the phosphorylation of hydrophobic site affects the stability of the activation loop site, so that chronic dephosphorylation of Ser473 in the stable transfectants likely impacts the phosphorylation state of Thr308.

To test whether PHLPP could inhibit the tumorigenicity of glioblastoma cells, LN229 cells stably expressing either the empty vector or HA-PHLPP were injected into nude mice, and the size of resulting tumors was monitored for 7 weeks. Injection of control LN229 cells resulted in formation of robust tumors, with a size of 940 $mm^3$ after 7 weeks (Figure 7C, open circles). Overexpression of PHLPP resulted in a striking 68% reduction in tumor size (n = 4, p value < 0.05), with the tumor in one mouse regressing completely (Figure 7C, open squares). Western blot analysis of dissected tumors revealed a marked reduction in phosphorylation of Ser473 in tumors from PHLPP-overexpressing cells compared to those from vector control LN229 cells (Figure 7D). Thus, reintroduction of PHLPP into tumor cells results in specific dephosphorylation of Akt at

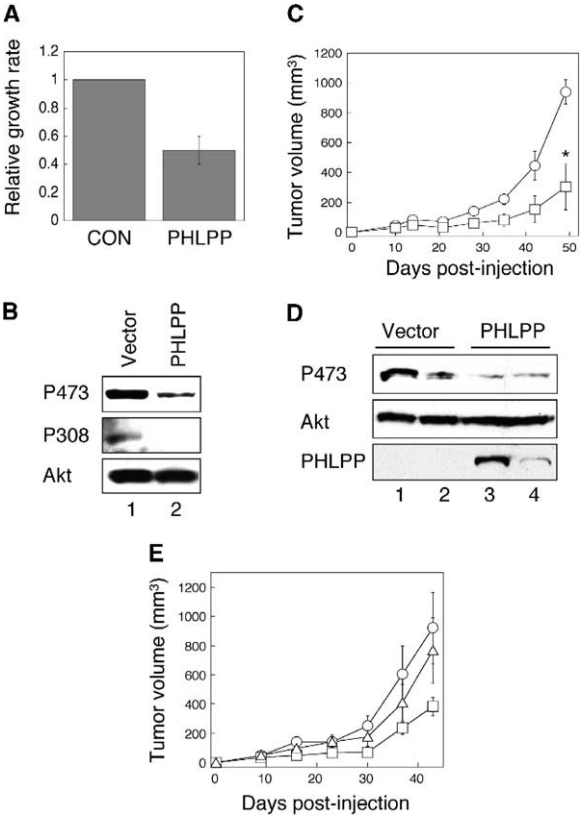


Figure 7. Effect of PHLPP on Proliferation and Tumorigenicity of Human Glioblastoma LN229 Cells

(A) LN229 cells were transfected with vector or HA-PHLPP. The number of viable cells in media containing G418 was determined by using a hemocytometer (Furnari et al., 1997). The growth rate of the vector-transfected cells (CON) was normalized to 1. The bar graph summarizes three independent experiments, showing the mean ± SEM (n = 3).

(B) Western blots showing phosphorylation of endogenous Akt in the G418-resistant LN229 cells. The stable LN229 cells expressing the empty vector (lane 1) or HA-PHLPP (lane 2) were analyzed by using P308, P473, and the anti-Akt antibody.

(C) Expression of PHLPP decreases tumorigenicity of LN229 cells in nude mice. Nude mice of BALB/c background were inoculated subcutaneously with stable LN229 cells ($3 \times 10^6$) transfected with either the empty vector (open circle) or HA-PHLPP (open square). The size of the tumors was measured every 7 days. Four mice were inoculated in each group, and data in the graph represent the mean ± SEM (n = 4, *p < 0.05).

(D) The phosphorylation status of Akt in tumors formed by LN229-injected nude mice. The tumor samples from two different mice either injected with the vector (lanes 1 and 2) or HA-PHLPP-transfected LN229 cells (lanes 3 and 4) were homogenized and analyzed with antibodies to phosphorylated Ser473 (top), total Akt (middle), or PHLPP (bottom).

(E) Deletion of the PDZ binding motif in PHLPP impairs its ability to suppress tumor growth in nude mice. Nude mice of BALB/c background were inoculated subcutaneously with stable LN229 cells ($5 \times 10^6$) transfected with either the empty vector (open circle), HA-PHLPP (open square), or HA-ΔC (open triangle). The size of the tumors was measured every 7 days. Four mice were inoculated in each group, and data in the graph represent the mean ± SEM (n = 4).

Ser473, suppression of cell proliferation, and, most importantly, suppression of tumor growth. Figure 7E shows that deletion of the PDZ binding motif of PHLPP almost completely abolishes the ability of PHLPP to reduce tumor size. These data reveal that the correct targeting of PHLPP is required for it to dephosphorylate Akt, promote apoptosis, and suppress tumors; its PP2C activity is not sufficient for the biological effects observed.

## Discussion

Misregulation of the PI3 kinase/Akt signaling pathway plays a central role in disease, most notably tumorigenesis. Hyperactivation of Akt tips the balance of cells into prosurvival pathways and is often correlated with tumor progression, whereas reduced activity of Akt tips the balance toward apoptosis and is often correlated with heart disease and diabetes (Lawlor and Alessi, 2001; Matsui et al., 2003). Much progress has been made in the past decade in elucidating the mechanisms that lead to Akt phosphorylation and activation (Hanada et al., 2004). In contrast, little is known about the mechanisms that terminate Akt signaling. Here, we report on the discovery of a phosphatase, PHLPP, that terminates Akt signaling by directly dephosphorylating the hydrophobic phosphorylation motif on Akt.

### Specificity of PHLPP

PHLPP displays remarkable discrimination between two phosphorylation sites within the same molecule: siRNA knockdown and overexpression studies reveal that it specifically dephosphorylates Ser473, but not Thr308, on Akt. Consistent with PHLPP specifically regulating Ser473, the phosphorylation state of this site is only modestly sensitive to OA, an inhibitor that does not affect PP2C family members. In contrast, the phosphorylation state of Thr308 is markedly sensitive to OA. Previous reports have shown that a PP2A-type activity regulates Akt (Andjelkovic et al., 1996; Yoshizaki et al., 2004); our data would suggest that the PP2A component regulates the PDK-1 site Thr308. It is noteworthy that in transient transfection (e.g., Figure 2A) or knockdown experiments (e.g., Figure 4B), PHLPP specifically modulates the phosphorylation state of Ser473 and not Thr308. However, in stable transfections of PHLPP (e.g., Figure 7B) and in cancer cell lines with chronically reduced PHLPP (e.g., Figure 6A), the phosphorylation state of both Thr308 and Ser473 is affected. This could arise from chronic (rather than acute) changes in phosphate on the hydrophobic motif regulating the long-term stability of phosphate on the activation loop. In this regard, phosphorylation of the hydrophobic motif stabilizes phosphorylation on the activation loop in protein kinase C (Bornancin and Parker, 1997; Edwards and Newton, 1997).

Whether there are other targets of PHLPP remains to be explored. However, the ability of a S473D construct of Akt to suppress the effect of PHLPP overexpression on apoptosis indicates that, in the studies presented, the biological effects derive primarily from the ability of PHLPP to dephosphorylate Ser473 of Akt. These effects are also specific for PHLPP; overexpression of another PP2C, PP2C$\alpha$, has recently been shown to have no effect on the hydrophobic motif of Akt (Yoshizaki et al., 2004).

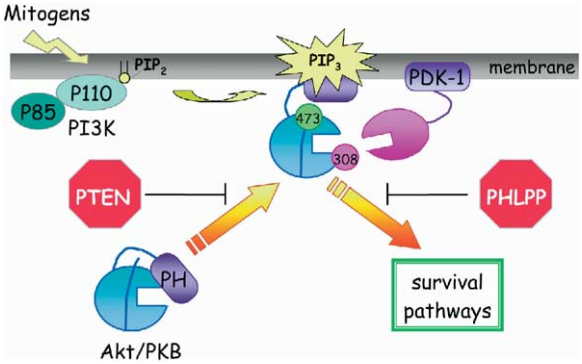


Figure 8. Model Showing Distinct Roles of a Lipid Phosphatase, PTEN, and a Protein Phosphatase, PHLPP, in Terminating Signaling by Akt

PTEN prevents Akt signaling by dephosphorylating the lipid second messenger Ptd InsP3. Here, we show that PHLPP terminates Akt signaling by dephosphorylating the hydrophobic motif, thus inactivating Akt. Misregulation of either promotes tumor progression.

### PDZ Binding Motif: Essential for Function

The PDZ binding motif is crucial for the biological function of PHLPP: deletion of the three carboxy-terminal residues encoding this motif inhibits the ability of PHLPP to dephosphorylate Akt, to promote apoptosis, and to suppress tumors. These data reveal that the correct intracellular targeting of PHLPP is critical for its function and suggest a scaffold that retains PHLPP in proximity to Akt. The PDZ binding motif is conserved in lower organisms such as *C. elegans* and *Drosophila* unlike the PH domain, which was added later in evolution and is not required for function.

### Potential Role of PHLPP in Cancer

PTEN has proven to be the archetypal tumor suppressor by its effects on the Akt signaling pathway. Nonetheless, there are abundant examples of Akt phosphorylation being elevated in cancer cell lines having wt PTEN (e.g., four out of five colon cancer cell lines screened in this study had elevated Akt phosphorylation and wt PTEN). Thus, it is clear that other mechanisms causing elevation of Akt phosphorylation contribute to tumor progression. Here, we show that PHLPP levels are markedly reduced in a number of colon cancer and glioblastoma cell lines. Reintroduction of PHLPP into a glioblastoma cell line that is wt in PTEN decreases the growth rate of these cells by ~50%. The magnitude of this growth-suppressive effect is similar to that observed after reintroduction of PTEN into glioblastoma cell lines defective in PTEN (Furnari et al., 1997). In addition, subcutaneous injection of glioblastoma cells transfected with PHLPP dramatically reduces the ability of these cells to induce tumors.

The chromosomal location of PHLPP on 18q21.33 poises it as an attractive candidate for a tumor suppressor, because this locus represents one of the most highly lost regions in colon cancer (Goel et al., 2003;

Jen et al., 1994). Indeed, assessment of 18q is a valuable predictor of colon cancer (Kelker et al., 1996).

Our data support a model in which the termination of Akt signaling is under the control of two key proteins: PTEN, a lipid phosphatase, that prevents activation by removing the second messenger that activates Akt, and PHLPP, a protein phosphatase, that inactivates Akt by direct dephosphorylation of the hydrophobic motif (Figure 8). A prediction of this model is that certain tumors will be insensitive to inhibitors of PI3 kinase or to overexpression of PTEN. This is indeed the case: the LN229 cells above are not sensitive to overexpression of PTEN and Akt phosphorylation in BT-474 breast cancer cells is resistant to LY29004 (Nicholson et al., 2003).

## Experimental Procedures

### Materials and Antibodies

Insulin, OA, and LY294002 were purchased from Calbiochem. PHLPP-specific siRNAs were obtained from Dharmacon. The targeting sequences in PHLPP for Si-2 and Si-3 are 5′-GGAATCAA CTGGTCACATT-3′ and 5′-ACTACTGGCAGGACACAAC-3′, respectively. *Drosophila* S2 cells and dsRNA against dPTEN were kindly provided by Dr. Jack Dixon. The following antibodies were purchased from Cell Signaling: phospho-antibodies for Thr308 (P308) and Ser473 (P473) of Akt, a phosphospecific antibody for the hydrophobic phosphorylation motif of PKC (P660), a phospho-antibody for Ser21/9 of GSK$\alpha/\beta$, a polyclonal Akt antibody, and a monoclonal Akt (5G3) antibody. A monoclonal antibody against GSK$\alpha/\beta$ was obtained from Biosource. An anti-HA monoclonal antibody was purchased from Covance. A PHLPP-specific antibody was raised against a His-tagged fusion protein of the C terminus of PHLPP (His-CT, amino acid residues 907–1205) (Strategic Biosolutions), and the antibodies were affinity purified against His-CT. The baculovirus encoding a 6His-tagged murine Akt1 was provided by Dr. Alex Toker.

### Molecular Cloning and Construction of Expression Plasmids

The full-length human PHLPP cDNA was cloned by combining the following cDNA fragments: KIAA0606 in Kazusa cDNA collection, an EST cDNA available from ATCC (GenBank accession number: BG110729), and a RT-PCR product using a mixture of human fetal brain cDNAs (Marathon-Ready cDNA from BD Biosciences Clontech) as the template. The 5′ end of PHLPP mRNA was confirmed by using the 5′-RACE method with human brain Marathon-Ready cDNA. The pcDNA3HA vector was created by inserting the cDNA coding sequence of an HA tag (MGYPYDVPDYA) into BamHI and EcoRI sites on the pcDNA3 vector. To express HA-tagged wt and a PH domain deletion mutant (ΔPH, deletion of amino acid residues 1–125) of PHLPP in mammalian cells, the corresponding cDNA fragments were amplified by using PCR. The PCR products were subcloned into EcoRI and XhoI sites on the pcDNA3HA vector to yield HA-PHLPP and HA-ΔPH expression constructs. The HA-ΔC PHLPP construct (deletion of the last three amino acid residues 1203–1205) was generated by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). To express the PP2C domain in bacteria, the coding region of PP2C (amino acid residues 653–906) was amplified by using PCR, and the PCR product was subcloned into EcoRI and XhoI sites on the pGEX-KG vector to yield in-frame fusion of the PP2C domain to GST.

### In Vitro Phosphatase Assay

A GST-fusion protein of PHLPP-PP2C was expressed in BL21 (DE3) and purified with glutathione-Sepharose. The GST-PP2C proteins were eluted from the beads in PBS containing 10 mM glutathione and 1 mM DTT and dialyzed against 50 mM Tris (pH 7.4) and 1 mM DTT. His-tagged Akt 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-Akt for 3 days. To obtain maximally phosphorylated Akt as substrate, the infected cells were treated with 10% fetal bovine serum (FBS) and calyculin A (100 nM) for 15 min prior to lysis. The infected cells were lysed in PBS containing 1% Triton X-100 and 10 mM imidazole, and His-Akt proteins were purified with 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 0–30 min. The final concentration of His-Akt and GST-PP2C in the reactions was 0.3 μM and 5 nM, respectively.

### Cell Transfection and Western Blotting

293T, H157, MDA-MB-231, LN229, LN319, and LN444 cells were maintained in DMEM (Cellgro) containing 10% FBS (Omega Scientific) and 1% penicillin/streptomycin at 37°C in 5% $CO_2$. DLD1 and HT29 cells were maintained in Iscove's MDM (Invitrogen) containing 10% FBS and 1% penicillin/streptomycin at 37°C in 5% $CO_2$. Transient transfection of all cell types was carried out by using Effectene reagents (Qiagen). Lipofectamine 2000 (Invitrogen) was used to transfect siRNAs into 293T and H157 cells. For Western blotting, the transfected cells were lysed 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, and 1 mM PMSF). The detergent-solublized cell lysate was obtained by centrifuging the whole cell lysate in a microcentrifuge at 13,000 rpm for 2 min. The detergent-solublized lysates were separated on SDS-PAGE gels. If multiple antibodies were used to probe the same set of lysates, equal amount of lysates were run on separate gels, and Western blotting was performed independently with each phospho-specific and total protein antibodies.

### Immunoprecipitation

To examine the interaction between PHLPP and Akt, 293T cells were transiently cotransfected with HA-PHLPP and a Flag-tagged Akt. ~30 hr posttransfection, the cells were lysed in buffer A. Coimmunoprecipitation was carried out by incubating the detergent-solublized cell lysates with an anti-HA monoclonal antibody and Ultra-link protein A/G beads (Pierce) at 4°C overnight. The immunoprecipitates were washed three times in buffer A. Bound proteins were separated on a SDS-PAGE gel and analyzed by using Western blotting.

### In Vitro Kinase Assay

H157 cells transfected with vector or HA-PHLPP were lysed in buffer A. The detergent-solublized cell lysates were incubated with an anti-Akt monoclonal antibody (5G3) and Ultra-link protein A/G beads (Pierce) at 4°C overnight. Beads were washed three times in buffer A and twice in buffer B (25 mM Tris, [pH 7.4], 10 mM $MgCl_2$ and 1 mM DTT). In vitro kinase assays were carried out by incubating immunoprecipitated Akt in buffer containing 25 mM Tris, (pH 7.4), 10 mM $MgCl_2$ and 1 mM DTT, 200 μM ATP, 6 μM Crosstide (Upstate Biotechnology) (Alessi et al., 1996), and $^{32}P\gamma$-ATP at 30°C for 30 min. Each reaction was performed in duplicate. The reactions were spotted onto P81 filter paper (Whatman), and radioactivity incorporated into the peptide was measured by using a scintillation counter.

### Apoptosis Assay

Apoptosis assays were performed as described previously (Brognard et al., 2001). Briefly, cells were transfected with different PHLPP expression constructs or siRNAs. In the case of gating for transfected cells, a GFP expression construct was cotransfected into the cells. ~24 hr posttransfection, the cells were switched to low-serum medium (DMEM plus 0.1% FBS) and allowed to grow for an additional 48 hr. For LY294002 (25 μM) treatment, the drug was added at the time when the cells were switched into the low-serum medium. The cells were collected and stained with propidium iodide (25 μg/ml). Apoptotic cells were defined as sub-2N DNA-containing cells. Quantification of apoptosis was obtained by using flow cytometry analysis with a Becton Dickinson FACSort.

### dsRNA Interference in *Drosophila* Cells

dsRNA interfernce in fly cells was performed essentially as described (Clemens et al., 2000). dPHLPP was identified in the fly gene database, and the predicted gene product resides on chro-

mosome 2L (gene CG10493, http://www.flybase.org). A fragment of dPHLPP cDNA was obtained by RT-PCR using total RNA from S2 cells as template, and the RT-PCR product was cloned into the pGEM-T vector (Promega). This construct was then used as a template for the subsequent PCR amplification. A 530 bp targeting sequence within dPHLPP was used to generate a PCR product containing a 5′ T7 RNA polymerase binding site, and the primers used are: 5′-TTAATACGACTCACTATAGGGAGACAGTTCAAGGTTTGTCAGAGC-3′ and 5′-TTAATACGACTCACTATAGGGAGATCCAGTGCTTGCCATGCG-3′. This PCR product was used as a template to produce dsRNA eoyj a MEGAscript T7 Transcription Kit (Ambion). S2 cells were maintained in Schneider's *Drosophila* medium (Invitrogen). To knockdown dPHLPP or dPTEN, 15 μg of dsRNAs were added to 2 × $10^6$ cells in a 6-well cell culture dish. 2 days after addition of dsRNAs, the cells were serum starved for 2 hr then treated with 300 nM insulin for 5 min. Detergent-solublized cell lysates were prepared and subjected to SDS-PAGE and Western blotting analysis.

### Tumorigenicity

LN229 cells were transfected with vector, HA-PHLPP, or HA-ΔC. ~30 hr posttransfection, the cells were switched to selection media (DMEM, 10% FBS, 1% penicillin/streptomycin, and 800 μg/ml G418). The viable cells were counted after 7 days in selection media. The number of viable cells reflects the effect of PHLPP on cell proliferation (Furnari et al., 1997). The G418-resistant stable cells were propagated and expanded for an additional 2–3 passages to obtain the number of cells needed for mice injection. For subcutaneous inoculation, 3 × $10^6$ or 5 × $10^6$ cells were injected into 4- to 5-week-old female nude mice of BALB/c background. The size of tumors was measured once a week with a caliper, and tumor volumes were defined as (longest diameter) × (shortest diameter)$^2$ × 0.5 (Nishikawa et al., 1994). Tumors were excised from individual mice and flash frozen in liquid $N_2$. The tumor samples were homogenized in buffer A, and detergent-solublized lysates were subjected to SDS-PAGE and Western blotting analysis.

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