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

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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

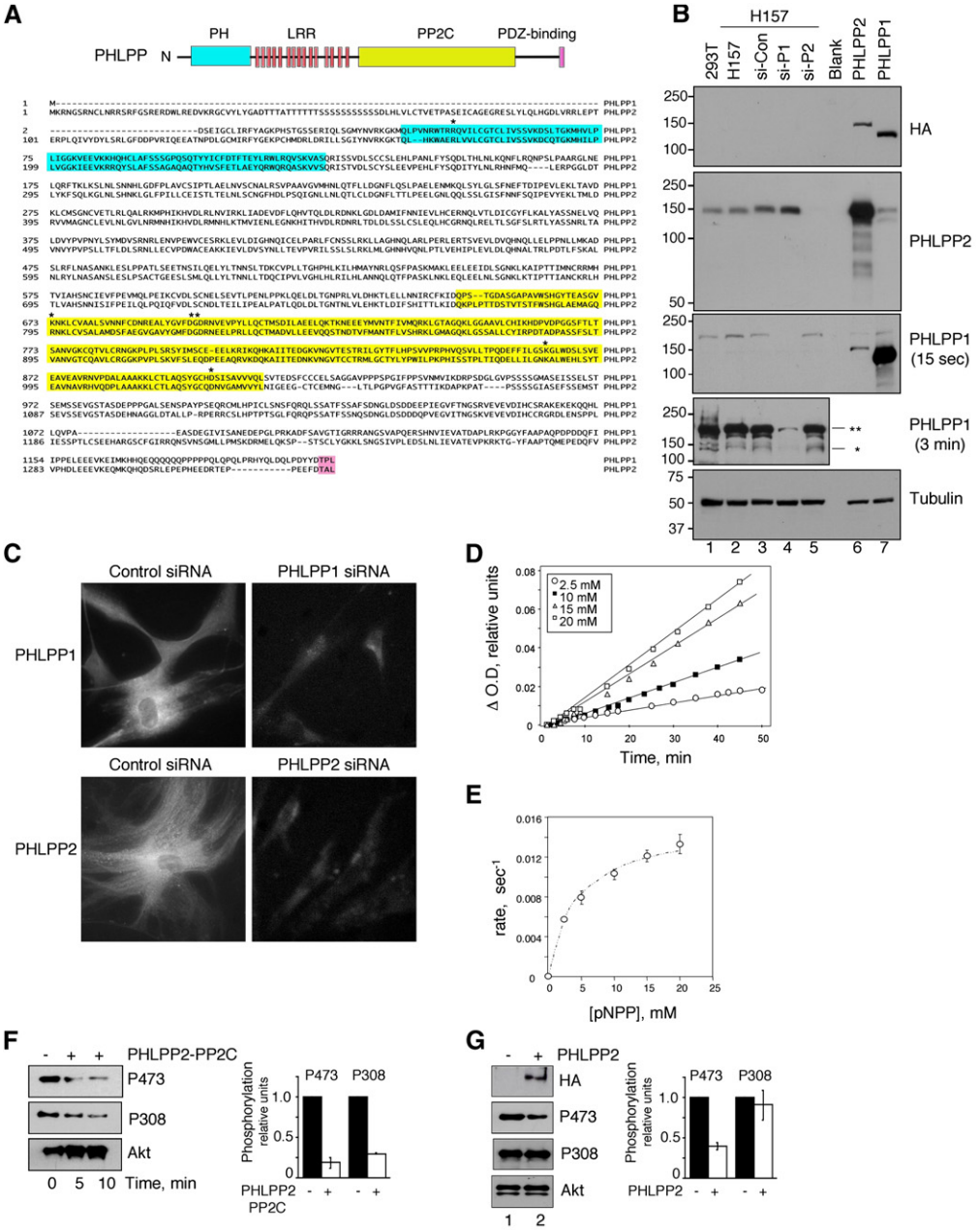

Figure 1. Domain Composition and Characterization of PHLPP2

(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.

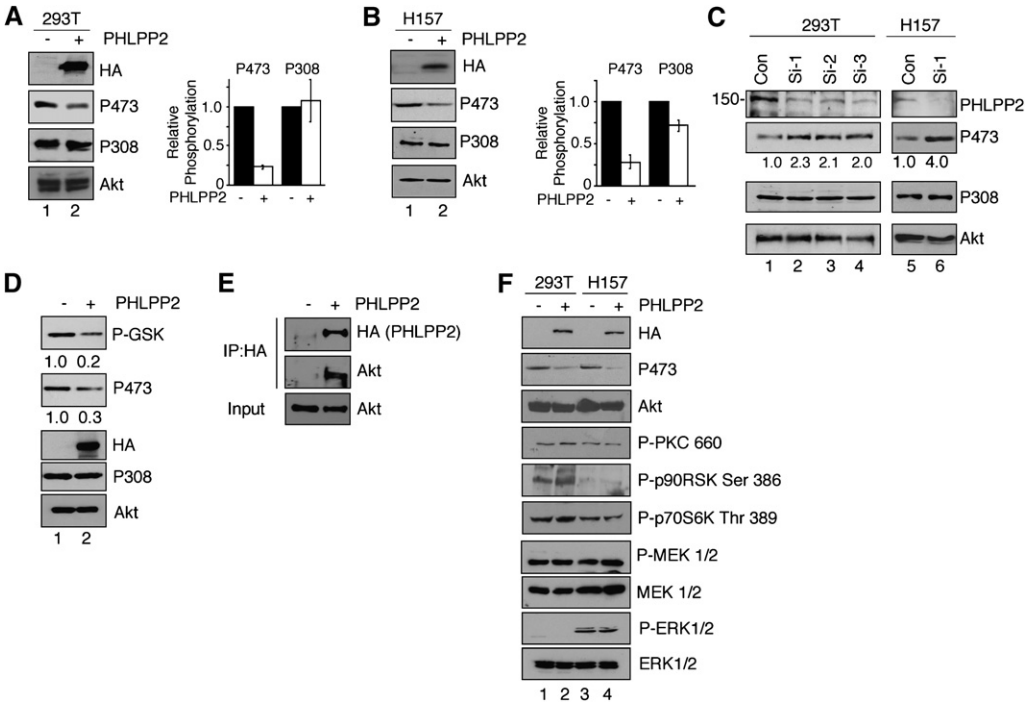

Figure 2. In Vivo Characterization of PHLPP2

(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

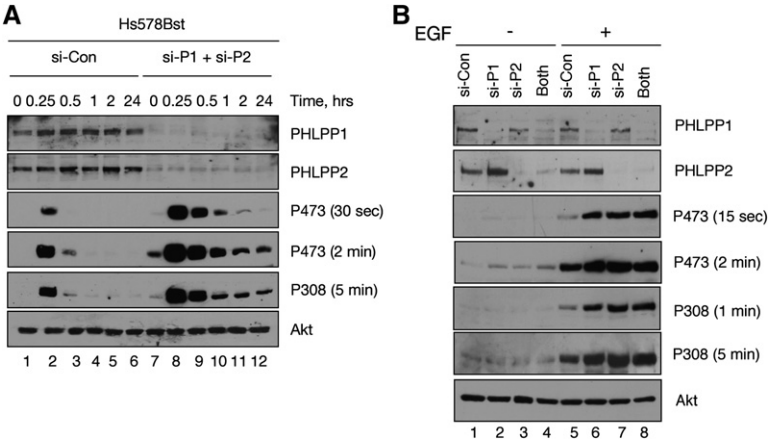

Figure 3. PHLPP Isoforms Control the Amplitude of Agonist-Stimulated Akt Phosphorylation

(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.

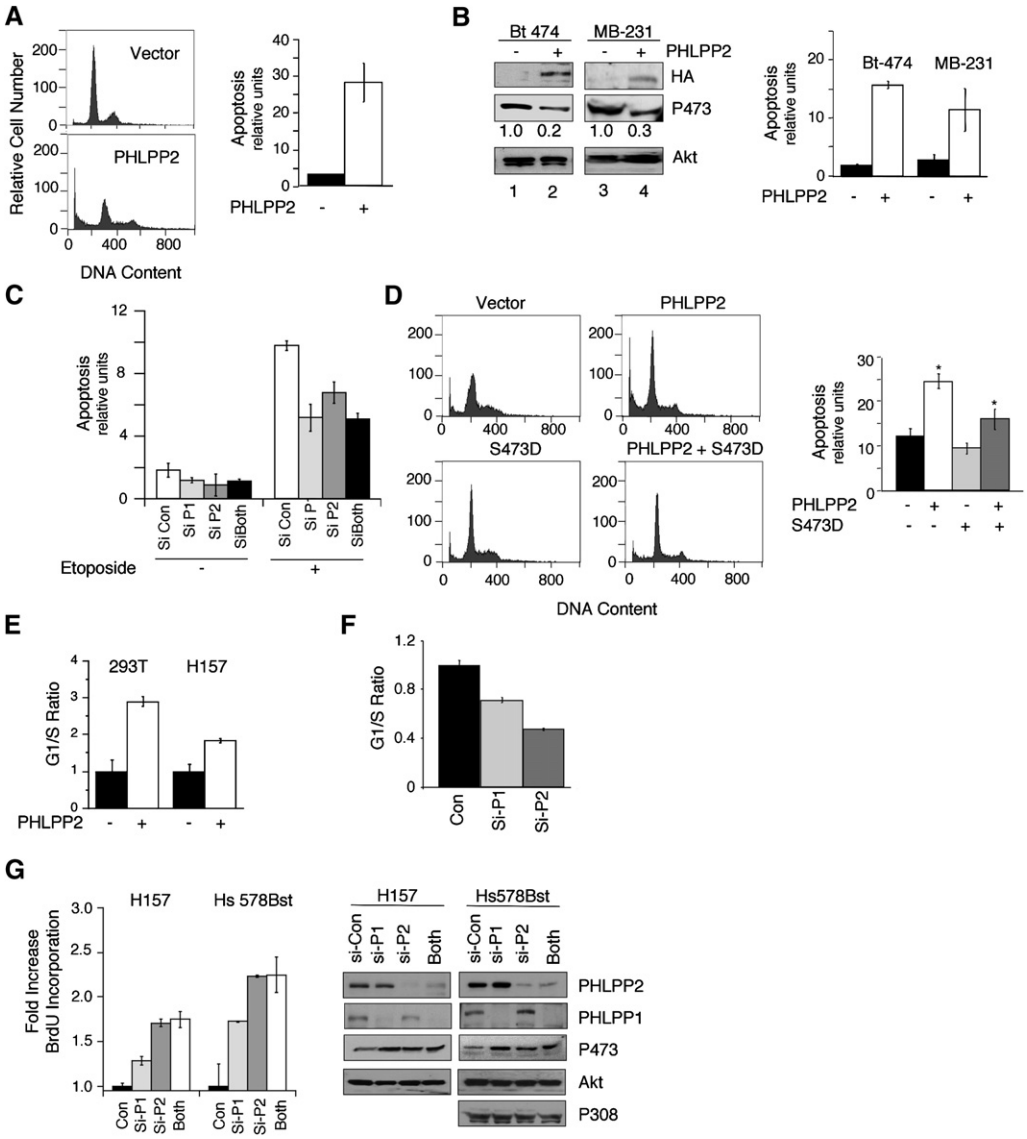

Figure 4. PHLPP2 Regulates Apoptosis and the Cell Cycle

(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.

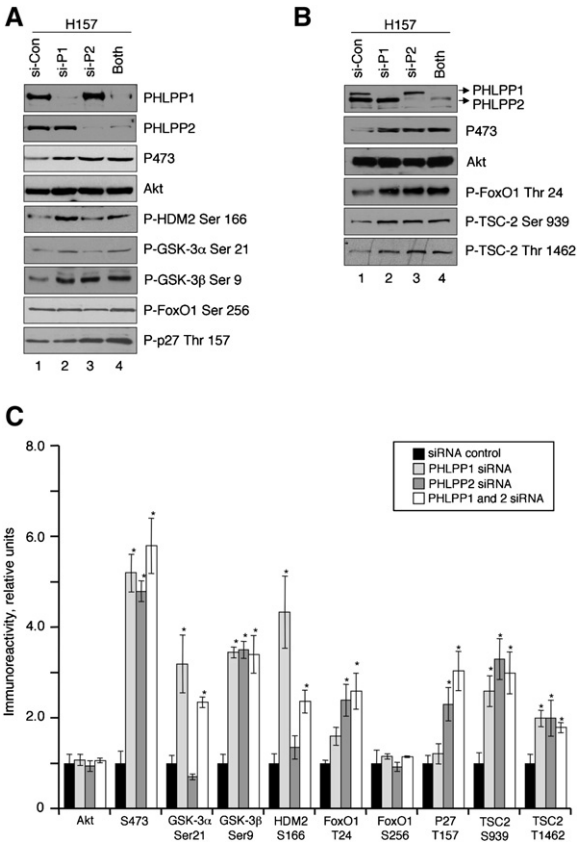

Figure 5. PHLPP1 and PHLPP2 Differentially Regulate Akt Downstream Substrates

(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

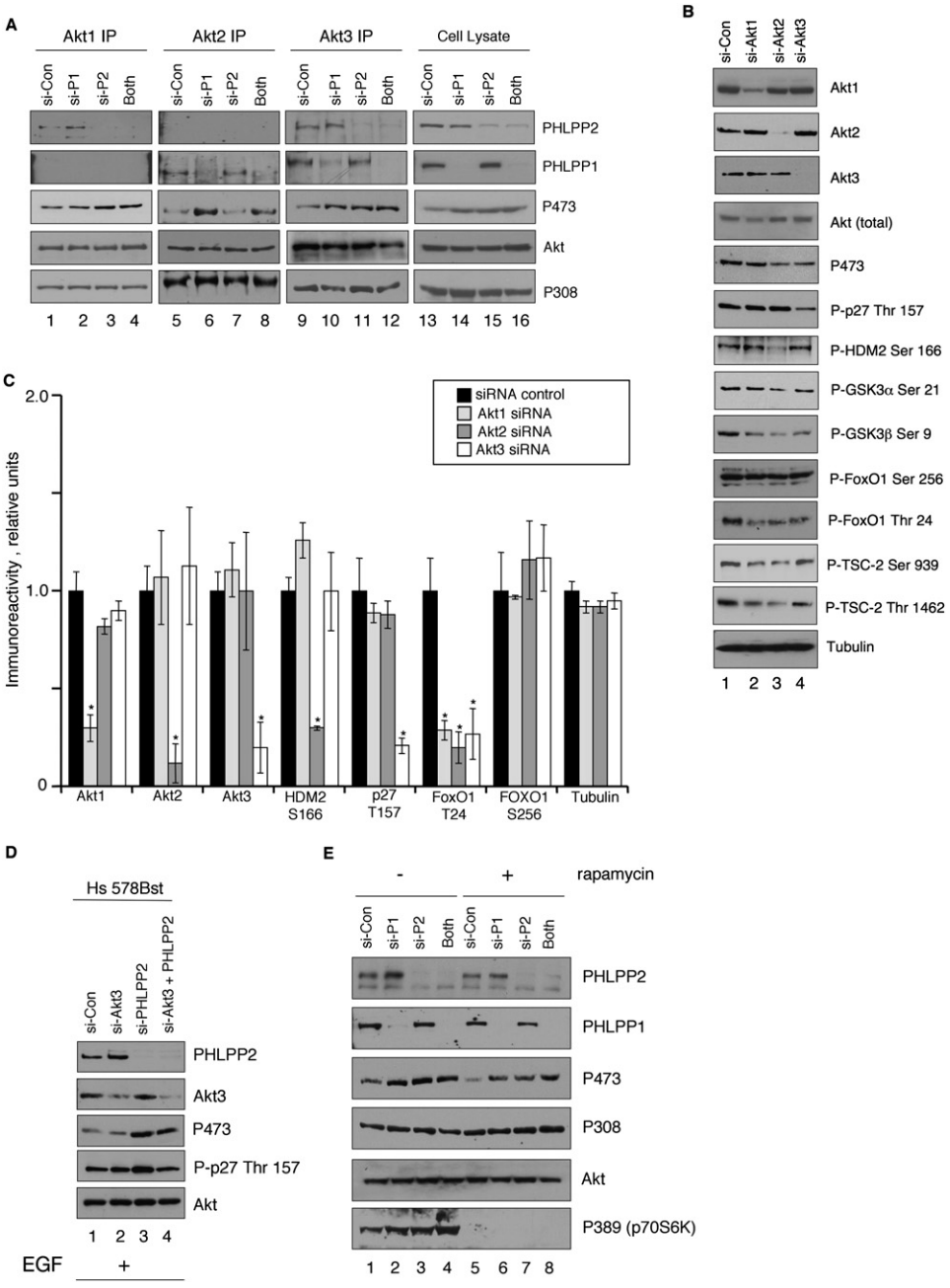

Figure 6. PHLPP1 and PHLPP2 Regulate Specific Akt Isoforms

(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.

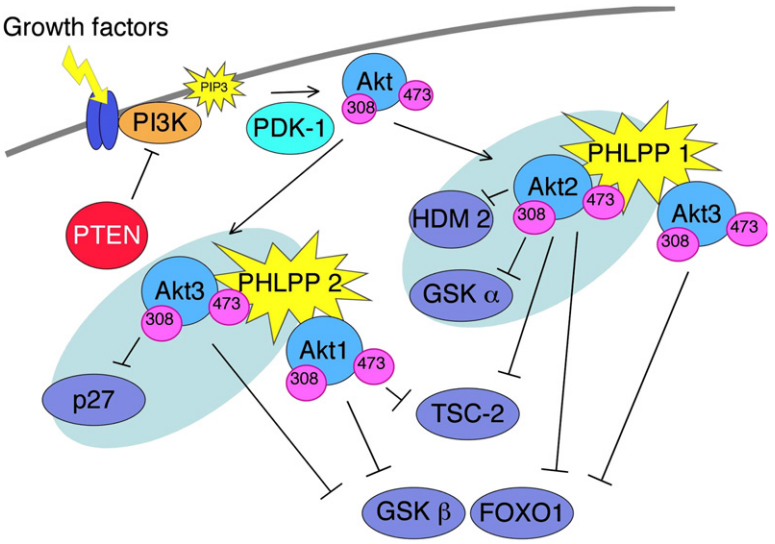

Figure 7. Model Illustrating How Specificity in Signal Termination by PHLPP Isoforms Is Achieved by Specific PHLPP-Akt-Substrate Complexes

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/.

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