β -TrCP-Mediated Ubiquitination and Degradation of PHLPP1 Are Negatively Regulated by Akt[∇]

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PHLPP1 belongs to a novel family of Ser/Thr protein phosphatases that serve as tumor suppressors by negatively regulating Akt signaling. Our recent studies have demonstrated that loss of PHLPP expression occurs at high frequency in colorectal cancer. In this study, we identified PHLPP1 as a proteolytic target of a β -TrCP-containing Skp–Cullin 1–F-box protein (SCF) complex (SCF ^{β -TrCP}) E3 ubiquitin ligase in a phosphorylation-dependent manner. Overexpression of wild-type but not Δ F-box mutant β -TrCP leads to decreased expression and increased ubiquitination of PHLPP1, whereas knockdown of endogenous β -TrCP has the opposite effect. In addition, we show that the β -TrCP-mediated degradation requires phosphorylation of PHLPP1 by casein kinase I and glycogen synthase kinase 3 β (GSK-3 β), and activation of the phosphatidylinositol 3-kinase/Akt pathway suppresses the degradation of PHLPP1 by inhibiting the GSK-3 β activity. Furthermore, expression of a degradation-deficient PHLPP1 mutant in colon cancer cells results in a more effective dephosphorylation of Akt and inhibition of cell growth. Taken together, our findings demonstrate a key role for β -TrCP in controlling the level of PHLPP1, and activation of Akt negatively regulates this degradation process.

Hyperactivation of phosphatidylinositol 3-kinase/Akt signaling is commonly associated with human cancers (1, 5, 27). Inability to terminate the growth and survival signals mediated by Akt is one of the major mechanisms contributing to the development of cancer (1, 22, 32). The activation of Akt involves two phosphorylation steps: it is first phosphorylated at the activation loop (Thr308) within the kinase core by PDK-1 and subsequently at the hydrophobic motif (Ser473) in the C terminus by the TORC2 complex (22). Since the activity of Akt is tightly controlled by phosphorylation, dephosphorylation of Akt leads to effective signaling termination by inactivating the kinase. Recently, a novel family of Ser/Thr protein phosphatases, PHLPP, has been identified to fulfill the role of a negative regulator for Akt via direct dephosphorylation (3, 14). Two isoforms of PHLPP, namely PHLPP1 and PHLPP2, are found in this phosphatase family. Although the two isoforms of PHLPP share their ability to dephosphorylate Akt, each PHLPP preferentially regulates a subset of Akt isoforms in human lung cancer cells (3). Several lines of evidence suggest that PHLPP functions as a tumor suppressor. For example, overexpression of PHLPP in glioblastoma and colon cancer cells inhibits tumorigenesis in xenografted nude mice (14, 20), while decreased PHLPP expression correlates with increased metastatic potential in breast cancer cells (26). Furthermore, our recent studies have shown that downregulation of both PHLPP isoforms occurs at high frequency in colorectal cancer clinical samples (20). Loss of tumor suppressor expression can be caused by alterations at the gene level such as loss of

heterozygosity or gene methylation. However, dysregulation of protein degradation pathways has also been implicated as a reason for downregulation of tumor suppressors (2, 6, 16).

The ubiquitin (Ub) proteasome pathway controls degradation of the majority of eukaryotic proteins (12). β -TrCP belongs to a large family of F-box-containing proteins, and it serves as the substrate recognition subunit in the SCF (Skp1–Cullin 1–F-box protein) Ub-E3 ligase protein complex (4). By regulating the proteolytic process of its substrates, β -TrCP plays an important role in controlling cell cycle and cancer biogenesis (10). It is believed that β -TrCP-mediated ubiquitination requires phosphorylation of its substrates (35). A consensus binding motif with the sequence of DSG(X)_{2–n}S (so-called “phospho-degron”) has been proposed, in which the two serine residues are phosphorylated prior to binding to β -TrCP (4). However, variations of this motif, including replacement of the serine residues with phosphomimetic residues (e.g., Glu or Asp) in the substrate sequence, have been shown to be equally effective in mediating association with β -TrCP (31, 34).

In this study, we report the identification of PHLPP1 as a proteolytic target of β -TrCP. We show that the degradation process of PHLPP1 depends on casein kinase I (CKI)- and glycogen synthase kinase 3 (GSK-3)-mediated phosphorylation, and activation of Akt negatively regulates PHLPP1 turnover. In addition, a PHLPP1 phosphorylation/degradation mutant antagonizes Akt more effectively in colon cancer cells.

MATERIALS AND METHODS

Reagents. The expression plasmids for Myc-tagged E3-ligases including β -TrCP1, Δ F-box/ β -TrCP1, FBW2, FBL5, Itch, and Myc-tagged GSK-3 β were gifts from Binhua Zhou (University of Kentucky). The Myc-CK1 α plasmid was generously provided by Wade Harper (Harvard Medical School). The mammalian expression constructs for glutathione S-transferase (GST)-tagged domains of PHLPP1 and the plasmid for expressing GST-PP2C of PHLPP1 in bacteria were generated as previously described (13, 14). All PHLPP1 mutant plasmids were

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generated by site-directed mutagenesis (Stratagene QuikChange kit) using pcDNA3-HA-PHLPP1 or GST-PP2C (14) as the templates. The following antibodies were purchased from commercial sources: polyclonal antibodies for PHLPP1 and Myc from Bethyl Laboratory; polyclonal antibodies against Akt, polyclonal phospho-Akt (p473) and phospho-GSK-3 α/β (Ser21/9) from Cell Signaling; a monoclonal antibody (MAB) for GSK-3 β from Biosource; an anti-hemagglutinin (HA) high-affinity rat MAB from Roche; an anti-myc MAB from Santa Cruz Biotechnology; and anti- γ -tubulin MAB from Sigma. β -TrCP1/2-specific small interfering RNA (siRNA) was synthesized by Invitrogen, and the targeting sequence was reported previously (7). Inhibitors including LY290042, CK1 inhibitor-D4476, GSK-3 inhibitor IX, and MG-132 were purchased from EMD/CalBiochem, and the concentrations of the inhibitors used are as follows: MG-132, 10 μ M; CK1-I, 10 μ M; GSK3-I, 30 nM; and LY294002, 20 μ M. [γ - 32 P]ATP (3,000 Ci mmol $^{-1}$) was obtained from PerkinElmer Life Sciences. The recombinant active forms of CK1, GSK-3 β , and lambda protein phosphatase (λ -PPase) were obtained from New England Biolabs.

Cells. Human colon cancer cells HCT116 and HCT-P1 (HCT116 cells overexpressing wild-type PHLPP1 [20]) were cultured in McCoy's 5A medium, and Caco2 and 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen). All media were supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin. Transient transfection of all cell types was carried out using Lipofectamine 2000 transfection reagents (Invitrogen). To knockdown β -TrCP1 and β -TrCP2 expression in cells, On-TARGETplus siRNAs specific for each gene were obtained from Dharmacon. A combination of siRNAs for both genes was used at total concentration of 50 nM to transfect cells. The short hairpin RNAs (shRNAs) for human Akt1, GSK-3 α , and GSK-3 β genes were constructed in pLKO.1-puro vector and purchased from Sigma-Aldrich (30), and the targeting sequences are as follows: Akt1, CGCGTGACC ATGAACGAGTTT; GSK-3 α , CCAGGACAAGAGGTTCAAGAA; and GSK-3 β , CCGATTGCGTTATTTCTCTA. The lentivirus-mediated delivery of shRNA and selection for stable knockdown cells were carried out as previously described (20). To generate stable cells overexpressing wild-type and mutant PHLPP1, the coding sequences of HA-PHLPP1 and HA-P1/4A were subcloned into pBabe-puro vector (Addgene), and the corresponding retroviruses were subsequently produced as described previously (23). HCT116 cells infected with the PHLPP1 retroviruses were subjected to selection with puromycin (2 μ g/ml).

CHX chase assay. Equal numbers of Caco2 or HCT116 stable cells were seeded onto 12-well plates and allowed to attached for $\sim$ 18 h. The cells were treated with cycloheximide (CHX; 20 μ g/ml) and harvested at indicated time points. The cell lysates were prepared in sodium dodecyl sulfate (SDS) sample buffer directly, and an equal amount of lysates was analyzed by immunoblotting. To examine the effects of different inhibitors, MG-132, CK1-I, or GSK3-I were added 30 min prior to the addition of CHX.

IP. Immunoprecipitation (IP) experiments were performed following procedures described previously (14). Briefly, the cells were lysed in buffer A (50 mM Na $_2$ HPO $_4$, 1 mM sodium pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol [DTT], 200 μ M benzimidazole, 40 μ g ml $^{-1}$ leupeptin, 200 μ M phenylmethylsulfonyl fluoride), and the detergent-solubilized cell lysates were incubated with the indicated antibodies and protein A/G agarose at 4°C for 3 h. The IP beads were washed twice in buffer A and twice in buffer B (buffer A plus 200 mM NaCl). Bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting. The density of Western blot signals was obtained and quantified using a FluorChem digital imaging system (Alpha Innotech).

In vivo ubiquitination of PHLPP1. A mammalian expression construct was generated to express PHLPP1 without any epitope tag. Briefly, the coding sequence of human PHLPP1 was amplified using PCR and subcloned into the EcoRI/XhoI sites on the pcDNA3 vector directly. To examine the ubiquitination of PHLPP1 under different treatments, 293T cells were cotransfected with expression constructs of HA-Ub and untagged PHLPP1. Approximately 40 h post-transfection, the cells were treated with the indicated inhibitors. To compare the ubiquitination of wild-type PHLPP1 with the mutants, HCT116 stable cells overexpressing wild-type PHLPP1 or the mutants were transfected with Myc-Ub expression plasmid. The transfected cells were lysed in buffer C (50 mM Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 10% Triton X-100, 1 mM DTT, 200 μ M benzimidazole, 40 μ g ml $^{-1}$ leupeptin, 200 μ M phenylmethylsulfonyl fluoride, and 10 mM *N*-ethylmaleimide). The detergent-solubilized cell lysate was incubated with the PHLPP1 antibody and protein A/G agarose at 4°C for 3 h. The IP beads were washed three times in buffer D (buffer C plus 250 mM NaCl) and once in buffer C. Bound proteins were analyzed by SDS-PAGE and immunoblotting.

MS and site-directed mutagenesis. Coomassie-stained SDS-PAGE gel bands containing either HA-PHLPP1 isolated from HCT-P1 cells (pretreated with a phosphatase inhibitor Calyculin A) or GST-PP2C fusion proteins phosphory-

lated with CK1 and GSK-3 β were excised and subjected to in-gel digestion and reversed-phase microcapillary liquid chromatography tandem mass spectrometry (MS/MS) using an LTQ 2D linear-ion-trap mass spectrometer (Thermo-Scientific). The data were obtained and analyzed at the Beth Israel Deaconess Medical Center Mass Spectrometry Core Facility. The following phospho-peptides were identified (the phosphorylated Ser and Thr residues are indicated by asterisks): PHVQ*SVLL*TPQDEFFILGSK and GLWDSL*SVVEAVEAVR. Mutations in PHLPP1 were created with the Quickchange site-directed mutagenesis kit (Stratagene).

In vitro phosphorylation and GST-pull-down assays. Recombinant GST-PP2C fusion proteins of wild-type and mutant PHLPP1 were expressed in bacteria and purified using glutathione-Sepharose as described previously (14). Purified GST-PP2C proteins were left bound on beads. The beads were washed three times with kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 10 mM MgCl $_2$, and 5 mM DTT). In vitro phosphorylation reactions were carried out by combining 40 μ l of glutathione-Sepharose-bound GST-PP2C in 10 μ l of kinase reaction buffer containing 10 μ Ci of [γ - 32 P]ATP and active CK1 and/or GSK-3 β (1 U per reaction). The samples were incubated at room temperature for 30 min, and the reactions were terminated by addition of SDS sample buffer. The phosphorylated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes for visualization by autoradiography.

To examine the direct binding between β -TrCP1 and GST-PP2C of PHLPP1, the TNT T7 coupled reticulocyte lysate system (Promega) was used to produce 35 S-labeled Myc-tagged β -TrCP1 using pcDNA3-Myc- β -TrCP1 as the template. The recombinant GST-PP2C proteins were purified and phosphorylated as described above, and phosphorylation reactions were terminated by adding buffer A. Subsequently, the GST-pull-down assays were carried out by incubating GST-PP2C-bound beads with 35 S-labeled Myc- β -TrCP1 at 4°C for 3 h. The beads were washed twice in buffer A and twice in buffer B, and the bound proteins were analyzed by SDS-PAGE and transferred to polyvinylidene difluoride membranes for visualization by autoradiography. The membranes were subsequently blotted with the anti-GST antibody to reveal the amount of GST-PP2C protein.

Detection of PHLPP1 phosphorylation at Ser847 using a phospho-specific antibody. A phospho-specific antibody for the S847 residue in PHLPP1 was generated against a phosphorylated peptide with the sequence FLHPSVVPRP HVQS $\underline{VLL}$ (in which the phospho-Ser847 residue is underlined). The phosphopeptide was conjugated to keyhole limpet hemocyanin and injected into rabbits at Bethyl Laboratories. The antibodies were affinity purified against the phosphopeptide; the resulting antibody is termed "P847." To detect the phosphorylation of PHLPP1, the cells were treated with dimethyl sulfoxide or MG-132 for 4 h followed by calyculin A (100 nM) for 20 min to trap the phosphorylated species. The cells were analyzed as described in the "IP" section above. The immunoprecipitated PHLPP1 proteins were separated by SDS-PAGE and subjected to immunoblotting analysis with P847.

Phosphatase treatment and β -TrCP binding. 293T cells transfected with HA-PHLPP1 and HA-P1/4A were lysed in buffer A. Two identical IPs were set up for both HA-PHLPP1 and HA-P1/4A, and each IP was carried out by incubating 1 mg of cell lysates with the PHLPP1 antibody and protein A/G-agarose at 4°C for 3 h. The IP beads were washed three times with buffer B and twice with λ -PPase reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM DTT, 0.1 mM EGTA, and 0.01% Brij 35). One set of the IP beads was then incubated with λ -PPase at room temperature for 30 min, while the other set was left untreated. Following the dephosphorylation reaction, the beads were washed three times in buffer A. 293T cells separately transfected with Myc- β -TrCP1 were lysed in buffer A, and equal amounts of the lysates were incubated with the washed IP beads from above at 4°C for 3 h. The IP beads were then washed twice in buffer A and twice in buffer B, and associated proteins were analyzed by SDS-PAGE and immunoblotting.

Cell proliferation assay. Equal numbers of HCT116 stable cells were seeded onto 12-well plates (2×10^4 cells/well) and allowed to grow for 4 days. For some cells, LY294002 was added to the media on the second day of the experiments. At the end of the experiments, the cells were fixed and stained with 0.5% crystal violet in 20% methanol for 30 min. After washing with water, the stained cells were dissolved in 1% SDS and A_{570} was determined.

RESULTS

PHLPP1 is downregulated by β -TrCP. To elucidate the potential mechanism of PHLPP degradation, we first examined whether PHLPP1 is downregulated via the proteasome pathway. Colon cancer Caco2 cells were treated with a protein

synthesis inhibitor, CHX, alone or in combination with the proteasome inhibitor MG-132, and the level of endogenous PHLPP1 was monitored for 8 h. PHLPP1 was effectively degraded in cells, and the degradation of PHLPP1 was blocked by MG-132 (Fig. 1A). Note that there are two splicing variants of PHLPP1, PHLPP1 α (the short form) and PHLPP1 β (the long form) (3), expressed in Caco2 cells. PHLPP1 β has a slightly faster degradation rate. Our present study focuses on PHLPP1 α as the cloning of PHLPP1 β cDNA has not been reported to date. However, the two splicing forms of PHLPP1 are likely to share a common degradation route as the majority of the protein sequences overlap between PHLPP1 α and PHLPP1 β . To keep the nomenclature consistent with previous reports, PHLPP1 α is simply referred as PHLPP1 (3, 14). A similar degradation rate was observed for overexpressed PHLPP1 in stable HCT-P1 cells (stable colon cancer HCT116 cells overexpressing PHLPP1 (20)) (Fig. 1B). The half-life of PHLPP1 is $\sim$ 4 h in both cell lines. These results provide initial evidence that PHLPP1 is degraded by the proteasome pathway in cells.

Since E3 ligases are known to bind their substrates with relatively high affinity, we screened a set of E3 ligases for their ability to interact with PHLPP1. Results from a representative co-IP experiment showed that PHLPP1 specifically associated with wild-type and Δ F-box mutant β -TrCP1 (a ubiquitination-deficient mutant in which the substrate binding moiety is intact) (Fig. 1C). As controls for specificity, little interaction was detected between PHLPP1 and other E3 ligases, including FBW2 (another F-box/WD40 repeat containing E3 ligase), FBL5 (an F-box/leucine-rich repeat containing E3 ligase), and Itch (a HECT domain E3-ligase). In addition, we found that β -TrCP2, a protein closely related to β -TrCP1, interacted with PHLPP1 as well (data not shown). Furthermore, the interaction between endogenous PHLPP1 and β -TrCP was detected in Caco2 cells (Fig. 1D).

To examine whether β -TrCP regulates the level of PHLPP1 in cells, we transfected HCT-P1 cells with wild-type or Δ F-box mutant β -TrCP1 and analyzed PHLPP1 expression. Overexpression of wild-type β -TrCP decreased PHLPP1 expression in HCT-P1 cells, while the Δ F-box mutant had the opposite effect (Fig. 1E). This is consistent with the notion that the Δ F-box mutant may serve as a dominant-negative construct by competing for substrate binding with endogenous β -TrCP (11, 18).

Knockdown of endogenous β -TrCP impairs PHLPP1 degradation. We next addressed whether endogenous β -TrCP plays a role in regulating PHLPP1 expression. Because of the functional redundancy between β -TrCP1 and β -TrCP2 (7, 24), we used a pool of siRNAs targeting both genes. Knockdown of endogenous β -TrCP resulted in a twofold increase in PHLPP1 expression in HCT-P1 cells (Fig. 2A). A similar increase in endogenous PHLPP1 expression was observed in Caco2 cells transfected with β -TrCP-specific siRNA (Fig. 2B). As a positive control, the expression of β -catenin, a known substrate of β -TrCP, was increased in the β -TrCP knockdown cells as well (Fig. 2B). Note that knockdown of either β -TrCP1 or β -TrCP2 by itself had very little effect on PHLPP1 expression (data not shown), confirming the functional redundancy of these two isoforms. Furthermore, we compared the rate of PHLPP1 degradation in cells transfected with β -TrCP-specific siRNA or the Δ F-box mutant to that in the control cells. As shown in Fig. 2C,

the half-life of PHLPP1 was increased from $\sim$ 4 h in the control cells to over 12 h in β -TrCP knockdown cells. Similarly, the rate of PHLPP1 degradation is decreased in cells overexpressing the Δ F-box mutant (Fig. 2D; the half-lives of PHLPP1 in the control and Δ F-box transfected cells are 4.2 and 8.2 h, respectively). Taken together, our findings demonstrate that the endogenous β -TrCP is involved in promoting the degradation of PHLPP1, and intact E3-ligase function is required for β -TrCP-mediated degradation.

β -TrCP promotes the ubiquitination of PHLPP1 in cells. To further assess whether β -TrCP is involved in the ubiquitination of PHLPP1, we performed *in vivo* ubiquitination experiments in transfected 293T cells. MG-132 was used to enrich the ubiquitinated species in cells. PHLPP1 was basally ubiquitinated, and treatment of cells with MG-132 increased the level of ubiquitination (Fig. 3A, lanes 1 and 2). Overexpression of wild-type β -TrCP significantly increased the amount of ubiquitination of PHLPP1 both basally and after MG-132 treatment, while the Δ F-box mutant had no effect (Fig. 3A, lanes 3 to 6). Furthermore, we analyzed the ubiquitination of PHLPP1 in β -TrCP knockdown cells. Since the ubiquitination of endogenous PHLPP1 was below the detection limit in these experiments, the expression constructs for PHLPP1 and HA-ubiquitin were cotransfected with the control or β -TrCP specific siRNA into 293T cells. As shown in Fig. 3B, a slight decrease of basal ubiquitination was observed in β -TrCP knockdown cells (compare lanes 1 and 3), and MG-132-induced increase of ubiquitination was also significantly blocked by depleting β -TrCP expression using RNA interference (compare lanes 2 and 4). Collectively, these results suggest that β -TrCP negatively regulates the stability of PHLPP1 by promoting its ubiquitination.

PHLPP1 is phosphorylated by CK1 and GSK-3 β in vitro and in cells. It has been shown previously that β -TrCP-mediated degradation is triggered by phosphorylation of its substrates (4, 35). Since β -TrCP targets a small phosphorylated motif in its substrates for binding, we first determined which domain of PHLPP1 interacts with β -TrCP in cells. The domain composition of PHLPP1 is shown in Fig. 4A. Different domains of PHLPP1 were constructed as GST-tagged fusion proteins, and GST-pull-down experiments showed that only the PP2C domain of PHLPP1 interacted with β -TrCP (Fig. 4B, lane 4). To search for potential kinases that may play a role in downregulating PHLPP1, we treated HCT-P1 cells with a panel of protein kinase inhibitors together with CHX for 8 h and monitored the level of PHLPP1 expression. Among all the kinase inhibitors examined, a CK1 inhibitor (CK1-I) and a GSK-3 inhibitor (GSK-3-I) were able to block the degradation of PHLPP1 (data not shown), suggesting that phosphorylation of PHLPP1 by CK1 and GSK-3 is likely required for degradation. Interestingly, both CK1 and GSK-3 are known to phosphorylate other β -TrCP substrates (17, 19, 36).

To identify the phospho-degrons for β -TrCP within the PP2C domain, PHLPP1 proteins were isolated from HCT-P1 cells by IP and subjected to phospho-peptide analysis by MS. In addition, the purified GST-PP2C fusion proteins were phosphorylated by CK1 and GSK-3 β *in vitro* and analyzed by MS as well. Three sites within the phosphatase domain, Ser847, Thr851, and Ser869, were found to be phosphorylated in cells and *in vitro* (see Materials and Methods). Moreover, we found

FIG. 1. β -TrCP promotes the degradation of PHLPP1. (A and B) Time course of PHLPP1 degradation. Caco2 (A) or stable HCT-P1 (B) cells were treated with CHX alone (lanes 1 to 6) or in combination with MG-132 (lanes 7 to 12) and chased for 0 to 8 h. Western blots were quantified using the FluoChem digital imaging system. The amount of PHLPP1 that remained was obtained by normalizing ECL enhanced chemiluminescence signals generated by the PHLPP1 antibody to that of tubulin, and the quantitative results were expressed graphically. Tubulin was used as the loading control (Con) throughout this study. The graphs in panel A and the left-side graph in panel B show the turnover of PHLPP1 with respect to time, and the graph on the right in panel B depicts the natural log of the percentage of PHLPP1 remaining. The data were fitted to a single exponential term by linear least-squares regression analysis ($r = 0.96$). The half-life of PHLPP1 was calculated to be 3.8 h in HCT-P1 cells. The rates of PHLPP1 decay were determined similarly throughout the study, and the half-lives of PHLPP1 were obtained by linear regression analysis. (C) PHLPP1 specifically interacts with β -TrCP1. 293T cells were cotransfected with HA-PHLPP1 together with one of the following plasmids: vector, Myc- β -TrCP1, Myc- Δ Fox/ β -TrCP1, Myc-FBW2, Myc-FBL5, or Myc-Itch (lanes 1 to 6, respectively). The cell lysates were immunoprecipitated with the anti-Myc MAb and analyzed by immunoblotting for PHLPP1. The presence of PHLPP1 in the immunoprecipitates and the cell lysates (10% input) was detected using the PHLPP1 antibody, while the Myc-tagged E3 ligases were detected by the anti-Myc polyclonal antibody. (D) Endogenous PHLPP1 and β -TrCP interact. Equal amounts of Caco2 cell lysates were incubated with either the protein A/G beads alone or the beads plus the PHLPP1 antibody (Ab; lanes 1 and 2, respectively). The immunoprecipitates were analyzed by immunoblotting for endogenous β -TrCP coimmunoprecipitated with PHLPP1. The presence of β -TrCP was detected using the β -TrCP MAb. The expression of PHLPP1 and β -TrCP in the cell lysates (10% input) is also shown. (E) Overexpression of wild-type β -TrCP1 promotes PHLPP1 degradation. HCT-P1 cells were transfected with vector, Myc- β -TrCP1, or Myc- Δ Fox/ β -TrCP1 (lanes 1 to 3, respectively), and the levels of PHLPP1 expression in the transfected cells were analyzed by immunoblotting. The expression of wild-type and mutant β -TrCP1 was detected using the anti-Myc MAb. The relative expression of PHLPP1 was obtained by normalizing the ECL signals generated by the PHLPP1 antibody to tubulin. The relative PHLPP1 level in the vector-transfected cells was set to 1. Data shown in the graph represent the mean $\pm$ standard error of the mean ($n = 4$). *, $P < 0.05$ as determined by two-sample t tests.

FIG. 2. Knockdown of endogenous β -TrCP expression inhibits PHLPP1 degradation. (A and B) PHLPP1 expression is elevated in β -TrCP knockdown cells. HCT-P1 (A) or Caco2 (B) cells were transfected with nontargeting control siRNA (Con-si) or β -TrCP-specific siRNA (si- β -Trcp) (50 nM; lanes 1 and 2, respectively), and the total cell lysates were analyzed for PHLPP1 and β -TrCP expression by immunoblotting. The expression of β -catenin in Caco2 cells was used as a positive control. The relative PHLPP1 expression was obtained by normalizing the amount of PHLPP1 to tubulin. The relative expression was indicated by the number below the PHLPP1 panel. (C) The rate of PHLPP1 degradation is decreased in β -TrCP knockdown cells. HCT-P1 cells transfected with the nontargeting control (lanes 1 to 6) or β -TrCP-specific siRNA (lanes 7 to 12) were treated with CHX, and the amount of PHLPP1 that remained at each time point was analyzed and quantified. Decreased expression of β -TrCP was detected in the knockdown cells using the β -TrCP MAb. (D) The rate of PHLPP1 degradation is decreased in Δ F-box-overexpressing cells. HCT-P1 cells transfected with control vector (lanes 1 to 6) or Δ F-box (lanes 7 to 12) were analyzed as in panel C. The expression of Δ F-box was detected using the anti-Myc antibody. Data shown in the graphs in panels C and D represent the linear regressions of the percentage of PHLPP1 that remained with respect to time.

that Ser867 and Ser869 are redundant in regarding to CK1-mediated phosphorylation. Thus, the Ser867 and Ser869 sites are mutated together in all subsequent experiments. These phosphorylation sites are termed the “N-clusters” and “C-clusters” (containing the Ser847/Thr851 and Ser867/Ser869 sites, respectively). The protein sequences surrounding these phosphorylation sites and the mutants created are shown in Fig. 4A.

To dissect the contribution of each phosphorylation site, the purified wild-type and mutant GST-PP2C fusion proteins were subjected to *in vitro* phosphorylation. The wild-type GST-PP2C was readily phosphorylated by CK1, while GSK-3 β was unable to induce any phosphorylation by itself. However, addition of CK1 enabled GSK-3 β to increase the total phosphorylation by 30% (Fig. 4C and D). This is consistent with the notion that GSK-3-mediated phosphorylation requires a priming phosphorylation of the substrate by other kinases (9). Interestingly, the amount of CK1-mediated phosphorylation was reduced by 50% in the C2A mutant, whereas addition of GSK-3 β together with CK1 resulted in a more pronounced increase over CK1 alone (Fig. 4D, lanes 4 to 6). Furthermore, mutation of all four phosphorylation sites led to a total loss of phosphorylation for both kinases (Fig. 4D, lanes 7 to 9). Based on the phosphorylation changes observed, we postulate that the PP2C domain of PHLPP1 is phosphorylated at two sites by CK1 and one additional site by GSK-3 β . Because CK1-mediated phosphorylation was reduced by half in the C2A mutant, either Ser867 or Ser869 likely constitutes one of the two CK1 sites. Since GSK-3 β requires a priming phosphate at the n + 4 position, Ser847 is most likely to be the GSK-3 β site, while

CK1-mediated phosphorylation of Thr851 provides the priming phosphate.

Between the two kinases regulating PHLPP1, CK1 is known to be a constitutively active kinase (25), while the activity of GSK-3 β is readily modulated in cells. To determine whether PHLPP1 is phosphorylated at the Ser847 site by GSK-3 β in cells, we generated a phospho-specific antibody against this site. As shown in Fig. 4E, the phosphorylated wild-type PHLPP1 was detected by the P847 antibody, while the antibody did not react with the S847A mutant. Furthermore, overexpression of GSK-3 β increased the amount of phospho-PHLPP1 detected in cells, whereas a GSK-3 β inhibitor, LiCl, eliminated phosphorylation of PHLPP1 at Ser847 (Fig. 4F). Interestingly, treatment of cells with MG-132 enriched the phosphorylated species of PHLPP1. Taken together, these results show that the Ser847 site in PHLPP1 is phosphorylated by GSK-3 β in cells, and PHLPP1 is rapidly targeted for proteasome-mediated degradation once phosphorylated.

Degradation of PHLPP1 requires CK1- and GSK-3-mediated phosphorylation. To further assess whether CK1 and GSK-3 activity is required for PHLPP1 degradation, we examined whether overexpression of CK1 α and GSK-3 β promotes PHLPP1 degradation in cells. Overexpression of CK1 α resulted in a marked decrease in wild-type PHLPP1 expression (Fig. 5A, lanes 1 and 2). However, this decrease was largely attenuated in both N2A and C2A mutants (lanes 3 to 6) and totally blocked in the 4A mutant (lanes 7 and 8). This is consistent with the *in vitro* phosphorylation results showing that each of the two clusters contains a CK1 targeting site, and

FIG. 3. Ubiquitination of PHLPP1 is mediated by β -TrCP in cells. (A) 293T cells were cotransfected with PHLPP1 and HA-Ub, together with vector (lanes 1 and 2), Myc- β -TrCP1 (lanes 3 and 4), or Myc- Δ Fox/ β -TrCP1 (lanes 5 and 6). The cells were treated with dimethyl sulfoxide or MG-132 for 2 h, and the cell lysates were immunoprecipitated with the PHLPP1 antibody. The ubiquitination of PHLPP1 was detected using the anti-HA rat MAb. The blot was stripped and reprobed with the PHLPP1 antibody to visualize total PHLPP1 in the immunoprecipitates. The expression of wild-type and mutant β -TrCP1 in the cell lysates was detected using the anti-Myc MAb. Con, control. (B) Knockdown of endogenous β -TrCP inhibits PHLPP1 ubiquitination. 293T cells were cotransfected with PHLPP1 and HA-Ub together with nontargeting control siRNA (Con-si; lanes 1 and 2) or β -TrCP-specific siRNA (si- β -Trcp; lanes 3 and 4) siRNA. The levels of PHLPP1 ubiquitination were assayed as described in panel A. Knockdown of β -TrCP expression was observed in the cell lysates (10% input). For both panels A and B, the relative ubiquitination was quantified by normalizing the level of ubiquitination (HA-Ub signals) to total PHLPP1 in the immunoprecipitates. The data shown in the graphs in both panels A and B represent the mean $\pm$ standard error of the mean ($n = 3$). *, $P < 0.05$.

two sites contribute together to increase PHLPP1 degradation. Similarly, overexpression of GSK-3 β reduced the expression of wild-type PHLPP1 and the C2A mutant (Fig. 5B, lanes 1 to 4). However, the addition of another mutation at either the GSK-3 β site (Ser847) or the priming site (Thr851) to C2A rendered it resistant to GSK-3 β (lanes 5 to 8). In addition, the 4A mutant was entirely insensitive to GSK-3 β overexpression (Fig. 5B, lanes 9 and 10).

To determine whether phosphorylation of Ser847 requires the priming phosphorylation at Thr851, we analyzed the phosphorylation status of 3A-1 and 3A-2 mutants using the P847 antibody. While the wild-type PHLPP1 was readily detected by the P847 antibody, a mutation at either the Ser847 or Thr851 site resulted in a complete loss of phosphorylation (Fig. 5C). This is consistent with the results shown in Fig. 5B that both mutants are resistant to GSK-3 β overexpression. Moreover, the N2A mutant is resistant to GSK-3 β overexpression as well (data not shown), confirming the requirement of phosphorylation at both Ser847 and Thr851 sites for GSK-3 β -mediated degradation of PHLPP1.

β -TrCP-mediated degradation of PHLPP1 depends on phosphorylation by CK-1 and GSK-3 β . To examine whether β -TrCP binds PHLPP1 in a phosphorylation-dependent manner, we performed in vitro GST-pull-down experiments. Purified GST-PP2C was first phosphorylated in vitro by CK1 and GSK-3 β , as described in Fig. 4C, and subsequently incubated with 35 S-labeled Myc- β -TrCP1 produced by in vitro transcription and translation. No specific interaction was detected between the unphosphorylated GST-PP2C and Myc- β -TrCP1 (Fig. 6A, lane 2). The amount of β -TrCP bound to GST-PP2C was increased upon phosphorylation by CK1, while addition of GSK-3 β alone had no effect (lanes 3 and 4), and a combination of both kinases further enhanced the interaction (lane 5). In contrast, the 4A mutant was unable to bind β -TrCP (lane 6). To further assess the phosphorylation status of PHLPP1 in cells and the effect of phosphorylation on β -TrCP binding, wild-type and mutant PHLPP1 proteins immunoprecipitated from transfected 293T cells were subjected to treatment with a nonselective phosphatase, λ -PPase. The control and phosphatase-treated PHLPP1 proteins were then incubated with

FIG. 4. PHLPP1 is phosphorylated by CK1 and GSK-3β. (A) Schematic representation of the domain composition of PHLPP1 and potential phosphorylation sites within the PP2C domain. The GST-PP2C protein comprises amino acid residues 642 to 910 of PHLPP1. Two clusters of phosphorylation sites, termed the “N-clusters” and “C-clusters,” are found in the PP2C domain of PHLPP1. The sequences surrounding the phosphorylation sites (shaded residues) are shown, and abbreviated names for individual phosphorylation mutants used in the study are listed. (B) The PP2C domain of PHLPP1 interacts with β-TrCP. The GST vector or GST-tagged different domains of PHLPP1, including PH, LRR, PP2C, and CT (lanes 1 to 5, respectively) were coexpressed with Myc-β-TrCP1 in 293T cells. The GST fusion proteins were pulled down using glutathione-Sepharose, and the precipitated proteins were analyzed by immunoblotting. The presence of β-TrCP1 bound to the beads and in the lysates (10% input) was detected using the anti-Myc MAb. The different GST fusion proteins precipitated were detected using the anti-GST antibody. (C) The PP2C domain of PHLPP1 is phosphorylated in vitro by CK1 and GSK-3β. The purified GST-PP2C fusion protein was subjected to in vitro phosphorylation by CK1, GSK-3β, or a combination of the two. The phosphorylated proteins were separated by SDS-PAGE and

FIG. 5. Degradation of PHLPP1 requires CK1 and GSK-3 activities. (A) CK1 α promotes PHLPP1 degradation. 293T cells were transfected with HA-PHLPP1 (wild type [WT]; lanes 1 and 2), HA-P1/N2A (lanes 3 and 4), HA-P1/C2A (lanes 5 and 6), or HA-P1/4A (lanes 7 and 8), together with either vector or Myc-CK1 α . The expression of PHLPP1 and CK1 α was analyzed by immunoblotting using the PHLPP1 and anti-Myc antibodies, respectively. (B) GSK-3 β promotes PHLPP1 degradation. 293T cells were transfected with HA-PHLPP1 (lanes 1 and 2), HA-P1/C2A (lanes 3 and 4), HA-P1/3A-1 (lanes 5 and 6), HA-P1/3A-2 (lanes 7 and 8), or HA-P1/4A (lanes 9 and 10), together with either vector or Myc-GSK-3 β . The expression of PHLPP1 and GSK-3 β was analyzed by immunoblotting using the PHLPP1 and GSK-3 antibodies, respectively. The relative expression levels were obtained by normalizing the amount of PHLPP1 to tubulin, and the level of wild-type PHLPP1 in the vector-transfected cells was set to 1. The data shown in the graphs in both panels A and B represent the mean $\pm$ standard error of the mean ($n = 3$). (C) Phosphorylation at Ser847 requires priming phosphorylation of Thr851. The HA-PHLPP1 (wild type; lanes 1 and 2), HA-P1/3A-1 (lanes 3 and 4), and HA-P1/3A-2 (lanes 5 and 6) expressed in 293T cells were immunoprecipitated and analyzed for phosphorylation at Ser847 using P847 antibody.

cell lysates overexpressing Myc- β -TrCP1 and analyzed for the amount of β -TrCP bound. Treatment of wild-type PHLPP1 with λ -PPase resulted in a marked decrease in β -TrCP binding (Fig. 6B, lanes 1 and 2). Less β -TrCP coimmunoprecipitated with the 4A mutant in the absence of λ -PPase, and the amount of β -TrCP bound to the 4A mutant was insensitive to the phosphatase treatment (lanes 3 and 4). Collectively, these re-

sults suggest that the binding between PHLPP1 and β -TrCP is controlled by the phosphorylation of the PP2C domain.

We next analyzed the expression of wild-type and mutant PHLPP1 in the presence of β -TrCP to determine whether β -TrCP-mediated degradation requires phosphorylation of PHLPP1 by CK1 and GSK-3 β . Coexpression of β -TrCP significantly decreased the expression of wild-type PHLPP1 (Fig.

analyzed by autoradiography (Autorad). (D) In vitro phosphorylation of mutant PP2C fusion proteins. The GST-PP2C (wild type [WT]; lanes 1 to 3), GST-PP2C/C2A (lanes 4 to 6), and GST-PP2C/4A (lanes 7 to 9) fusion proteins were phosphorylated and analyzed as in panel C. The relative phosphorylation of PP2C was obtained by normalizing the 32 P signals obtained from autoradiograph to the amount of GST fusion proteins, and this number for CK1-phosphorylated wild-type PP2C was set to 1. Phosphorylation of PP2C under all other conditions was normalized accordingly, and the numbers are indicated below the 32 P panel. Con, control. (E) PHLPP1 is phosphorylated at Ser847 in cells. The HA-PHLPP1 (wild type; lanes 1 and 2) and HA-P1/S847A mutant (lanes 3 and 4) PHLPP1 expressed in 293T cells were immunoprecipitated with the anti-PHLPP1 antibody and analyzed using P847 antibody. (F) Phosphorylation at Ser847 is controlled by GSK-3 β . 293T cells were cotransfected with HA-PHLPP1 together with either vector (lanes 1 and 2 and 5 and 6) or Myc-GSK3 β . One set of vector-cotransfected cells was treated with LiCl (10 mM) for 2 h (lanes 5 and 6). The immunoprecipitated PHLPP1 proteins were analyzed for phosphorylation at Ser847 using P847 antibody.

FIG. 6. β -TrCP-mediated degradation of PHLPP1 depends on phosphorylation of PHLPP1 by CK1 and GSK-3. (A) Binding of β -TrCP to GST-PP2C is phosphorylation dependent. Purified wild-type (WT) GST-PP2C fusion proteins were subjected to one of the following treatments: left untreated, phosphorylated by CK1, phosphorylated by GSK-3 β , or phosphorylated by a combination of the two kinases (lanes 2 to 5, respectively). The GST-PP2C/4A mutant proteins were phosphorylated with the combination of the two kinases as well (lane 6). Subsequently, the GST fusion proteins were incubated with ³⁵S-labeled Myc- β -TrCP1 and the bound β -TrCP1 was detected by autoradiography (Autorad). The amount of Myc- β -TrCP1 in the 10% input is also shown (lane 1). (B) Dephosphorylation of PHLPP1 reduces its binding affinity for β -TrCP. 293T cells expressing HA-PHLPP1 (WT; lanes 1 and 2) or HA-P1/4A (lanes 3 and 4) were lysed and immunoprecipitated with the PHLPP1 antibody. The immunoprecipitates were then treated with λ -PPase or left untreated followed by incubation with lysates of 293T cells overexpressing Myc- β -TrCP1. PHLPP1 and β -TrCP1 in the immunoprecipitates were detected using the PHLPP1 and anti-Myc antibodies. The amount of β -TrCP1 in the 10% input is shown as well. The relative β -TrCP1 binding was quantified by normalizing the amount of β -TrCP1 to PHLPP1 found in the immunoprecipitates. Data shown in the graph represent the mean $\pm$ standard error of the mean ($n = 3$). (C) β -TrCP-mediated degradation of PHLPP1 mutants. 293T cells were transfected with HA-PHLPP1 (wild type; lanes 1 and 2), HA-P1/N2A (lanes 3 and 4), HA-P1/C2A (lanes 5 and 6), or HA-P1/4A (lanes 7 and 8), together with either vector or Myc- β -TrCP1. The expression of PHLPP1 and β -TrCP1 was analyzed in the cell lysates using the PHLPP1 and anti-Myc antibodies, respectively. The relative expression levels were obtained by normalizing the amount of PHLPP1 to tubulin, and the level of wild-type PHLPP1 in the vector-transfected cells was set to 1. The data shown in the graph represent the mean $\pm$ standard error of the mean ($n = 3$).

6C, lanes 1 and 2). In contrast, the N2A and C2A mutants became partially resistant (lanes 3 to 6), while the 4A mutant was entirely insensitive to β -TrCP overexpression (lanes 7 and 8). In addition, we examined the effect of β -TrCP on the expression of phosphomimetic mutations of PHLPP1. Overexpression of β -TrCP resulted in decreased expression of all phosphomimetic mutants (including N2D, C2D, and 4D), and the level of reduction was similar to that observed in wild-type PHLPP1 (data not shown). These results are consistent with the notion that each of the phosphorylation clusters provides a binding site for β -TrCP and elimination of both sites is necessary to fully protect PHLPP1.

A degradation-resistant mutant PHLPP1 functions more effectively in cells. To better compare the differences between wild-type PHLPP1 and the mutants, stable cell lines overexpressing wild-type PHLPP1 and the 4A mutant were generated in HCT116 cells. CHX chase experiments showed that the rate of degradation was markedly reduced in the 4A mutant, and no detectable decay of the 4A mutant was observed during the course of the experiment (Fig. 7A). Thus, the 4A mutant of PHLPP1 is considered degradation resistant. Moreover, the level of ubiquitination in the 4A mutant was reduced basally, and overexpression of β -TrCP was unable to rescue the ubiquitination (Fig. 7B). These results confirm that the β -TrCP-

FIG. 7. Phosphorylation of PHLPP1 is required for degradation. (A) Phosphorylation of PHLPP1 is required for degradation. Stable HCT116 cells overexpressing HA-PHLPP1 (wild type [WT]; lanes 1 to 6) or HA-P1/4A (4A; lanes 7 to 12) were treated with CHX, and the rate of PHLPP1 degradation was monitored using the whole-cell lysates. The amount of PHLPP1 that remained compared to that at the initial time point (time zero) was quantified and is expressed graphically. (B) Ubiquitination of PHLPP1 mutants. Stable HCT116 cells overexpressing HA-PHLPP1 (wild type; lanes 1 and 2) and HA-P1/4A (4A, lanes 3 and 4) were cotransfected with a Myc-tagged Ub together with vector or Flag- β -TrCP1. The cell lysates were immunoprecipitated with the PHLPP1 antibody. The level of PHLPP1 ubiquitination was detected using the anti-Myc MAb. The blot was stripped and reprobed to visualize total PHLPP1 in the immunoprecipitates. The expression of Flag- β -TrCP1 in the lysates was detected by the anti-Flag MAb. (C) The 4A mutant of PHLPP1 is a more effective phosphatase. Stable HCT116 cells overexpressing vector (control [Con]), HA-PHLPP1 (wild type), or HA-P1/4A (4A) (lanes 1 to 3, respectively) were lysed and subjected to immunoblot analysis. The phosphorylation of Akt at the Ser473 site and the expression of PHLPP1, Akt, and tubulin were detected using the p473, PHLPP1, anti-Akt, and γ -tubulin antibodies. The amount of Akt phosphorylation relative to the control sample is indicated below the p473 panel. (D) Graph showing the rate of cell growth in stable HCT116 cells. Equal numbers of control (Con), wild-type, and 4A cells were seeded into regular growth media in the absence or presence of LY294002 (20 μ M) and allowed to grow for 4 days. The relative cell growth was set to 1 for the control cells in the absence of LY294002 (LY), and the cells in all other settings were normalized accordingly. Each experimental point was done in duplicates, and the graph represents the mean $\pm$ standard error of the mean of three independent experiments. (E) Inverse correlation of PHLPP1 and β -TrCP expression in colon cancer cells. Cell lysates prepared from colon cancer cells including Dld1, HT29, HCT116, KM12C, and KM12L4A (lanes 1 to 5, respectively) were analyzed by immunoblotting. The expression of PHLPP1 and β -TrCP was detected using the PHLPP1 antibody and the anti- β -TrCP MAb, respectively.

mediated ubiquitination depends on phosphorylation of PHLPP1.

Since PHLPP1 serves as a tumor suppressor by dephosphorylating Akt (14, 20), we examined whether the 4A mutant

functions more effectively at negatively regulating Akt. In the stable HCT116 cells, the basal expression of the 4A mutant was twofold higher than that of wild-type PHLPP1 (Fig. 7C). Overexpression of wild-type PHLPP1 resulted in a 60% decrease in

Akt phosphorylation at the Ser473 residue, while Akt was dephosphorylated to 25% of the control level in cells expressing the 4A mutant (Fig. 7C). To establish a functional link between PHLPP1 degradation and its ability to inhibit tumor cell growth, the rate of cell proliferation was determined in the stable cells. Our previous studies have shown that overexpression of wild-type PHLPP1 inhibits cell proliferation both basally and synergistically with LY294002 (20). While overexpression of wild-type PHLPP1 resulted in a 20% inhibition of cell growth, the 4A mutant reduced cell growth by 40% under basal conditions (Fig. 7D). Furthermore, LY294002-induced growth inhibition was further potentiated in cells expressing wild-type PHLPP1 and the 4A mutant. However, the 4A mutant exhibited a twofold-higher efficacy than the wild-type PHLPP1 (Fig. 7D). Thus, the 4A mutant of PHLPP1 antagonizes Akt more effectively due to higher expression in cells.

We have previously shown that loss of PHLPP1 expression occurs at high frequency in colon cancer clinical specimens (20). To determine whether increased the expression of β -TrCP is related to PHLPP1 downregulation, we analyzed PHLPP1 expression in colon cancer cell lines. Among the five cell lines tested, an inverse correlation between PHLPP1 expression and the level of β -TrCP protein was observed (Fig. 7E). Taken together, our results suggest that alteration of PHLPP1 expression is tightly associated with β -TrCP levels in cancer cells.

Degradation of PHLPP1 is negatively regulated by Akt. Since the GSK-3 β activity is negatively regulated by its upstream kinase Akt, we next examined whether Akt is involved in controlling PHLPP1 degradation. Stable knockdown cells were generated using lentivirus-based shRNA targeting Akt1. To exclude the potential involvement of Akt in modulating the mRNA level of PHLPP1, HCT-P1 cells were used as the parental cells in which the transcription of PHLPP1 is initiated from an exogenous cytomegalovirus promoter. CHX-chase experiments showed that the rate of PHLPP1 degradation was largely accelerated in Akt1 knockdown cells. Specifically, the half-life of PHLPP1 was reduced from ~4 h in the control cells to ~2 h in Akt1 knockdown cells (Fig. 8A). In addition, while the Akt-mediated inhibitory phosphorylation of GSK-3 was reduced in Akt1 knockdown cells, the level of PHLPP1 expression was also decreased to 25% of the control level (Fig. 8B). Conversely, overexpression of a constitutively active Akt (myristoylated-Akt1) markedly increased expression of PHLPP1 by promoting the inhibitory phosphorylation on GSK-3 β (Fig. 8C).

To further elucidate which GSK-3 isozyme is important in regulating PHLPP1 expression, we infected HCT-P1 cells with lentivirus-based shRNAs targeting either GSK-3 α or GSK-3 β . The PHLPP1 expression was markedly increased in the GSK-3 β stable knockdown cells, while knocking down GSK-3 α had no effect (Fig. 8D), suggesting that GSK-3 β is the major isoform responsible for PHLPP1 degradation. Moreover, the amount of PHLPP1 ubiquitination was elevated in Akt1 knockdown cells (Fig. 8E). Therefore, by negatively regulating GSK-3 β activity in cells, Akt plays a critical role in maintaining the stability of PHLPP1.

In summary, our results demonstrate that the degradation of PHLPP1 is controlled by a dual-phosphorylation mechanism (Fig. 8F). Two kinases, CK1 and GSK-3 β , phosphorylate PHLPP1 and provide two targeting sites for β -TrCP-mediated

ubiquitination and degradation. In addition, this downregulation of PHLPP1 is negatively regulated by its substrate Akt.

DISCUSSION

Recent studies have suggested that enhanced protein degradation may contribute to loss of tumor suppressor proteins in cancer (7, 12, 33). In this study, we investigate the mechanism by which PHLPP1, a novel protein phosphatase and tumor suppressor, is downregulated. Our results show that PHLPP1 specifically interacts with β -TrCP, a substrate binding subunit of SCF E3 ubiquitin ligase, and the levels of PHLPP1 expression and ubiquitination are tightly controlled by β -TrCP in cells. This β -TrCP-mediated degradation is promoted by CK1- and GSK-3 β -induced phosphorylation within the phosphatase domain of PHLPP1. More importantly, a phosphorylation/degradation-deficient mutant PHLPP1 is expressed at a higher level in colon cancer cells and functions more effectively at inhibiting cell growth. Furthermore, we show that inhibition of Akt activity accelerates PHLPP1 degradation and increases the ubiquitination of PHLPP1. Taken together, our findings demonstrate a key role of Akt in regulating the stability of its negative regulator, PHLPP1.

Phosphorylation of PHLPP1. In this study, we show that PHLPP1 is phosphorylated by CK1 and GSK-3 β and that phosphorylated PHLPP1 becomes a substrate targeted by β -TrCP for degradation. There are several lines of evidence indicating that the level of PHLPP1 is controlled by phosphorylation in cells: (i) PHLPP1 expressed in cells interacts with β -TrCP, and the binding decreases upon dephosphorylation of PHLPP1; (ii) the phosphorylation-deficient mutant (4A) is expressed at an elevated level in cells and is no longer regulated by β -TrCP; and (iii) phosphorylation of PHLPP1 by GSK-3 β at the Ser847 site is detected by the phospho-specific antibody, and this phosphorylated species is stabilized by the proteasome inhibitor. Our results show that phosphorylation of both the N- and C-clusters is required for β -TrCP-mediated degradation of PHLPP1. Based on the phosphorylation site analysis above, the sequences surrounding the N- and C-clusters (⁸⁴⁶QpSVLLpT⁸⁵¹ and ⁸⁶⁶LpSVEE⁸⁷², respectively) likely form the destruction motifs of β -TrCP. Although these two phospho-motifs (in bold-face) found in PHLPP1 do not match the canonical consensus, they meet the requirement of two positively charged/phosphorylated residues for binding β -TrCP. Interestingly, several recently identified β -TrCP substrates all lack the canonical consensus sequences (28, 31, 34), suggesting that β -TrCP is capable of recognizing various sequence deviations for degradation. Future studies are needed to depict that β -TrCP is directly involved in the ubiquitination of PHLPP1.

Our results indicate that mutation of either the Ser867 or Ser869 site alone does not decrease CK1-mediated phosphorylation in vitro, and these single-site mutants behave exactly the same as wild-type PHLPP1 with regard to degradation (data not shown). However, the stoichiometric analysis and MS analysis suggest that Ser869 is likely preferred by CK1 in the context of wild-type PHLPP1. It has been shown that CK1-mediated phosphorylation of the adenomatous polyposis coli protein occurs at Ser1510 within a stretch of sequence containing LpSLDE, in which the two acidic residues (underlined) are required for CK1 recognition. This type of sequence is referred

FIG. 8. Degradation of PHLPP1 is negatively controlled by Akt activity. (A) Degradation of PHLPP1 is accelerated in Akt1 knockdown cells. Stable control (shRNA control [sh-Con]; lanes 1 to 6) and Akt1 (Akt1 shRNA [sh-Akt1]; lanes 7 to 12) knockdown cells were treated with CHX, and the degradation of PHLPP1 was monitored using the whole-cell lysates. (B) PHLPP1 expression is decreased in Akt1 knockdown cells. The cell lysates prepared from stable knockdown cells (sh-Con and sh-Akt1; lanes 1 and 2, respectively) were analyzed for PHLPP1 expression and Akt and GSK-3 phosphorylation. The numbers shown below the PHLPP1 panel represent the relative expression of PHLPP1 in these cells. Note that the endogenous Akt2 expressed in the cells is detected by the P473 antibody in the Akt1 knockdown cells. (C) PHLPP1 expression is increased in Myr-Akt1-overexpressing cells. 293T cells cotransfected with HA-PHPP1 together with vector or HA-Myr-Akt were analyzed for PHLPP1 and Myr-Akt1 expression and GSK-3 phosphorylation. The numbers shown below the PHLPP1 panel represent the relative expression of PHLPP1 in these cells. (D) GSK-3 β is the major isoform regulating PHLPP1 expression. The cell lysates prepared from stable knockdown cells (sh-Con, GSK-3 α shRNA [sh-GSK-3 α], and sh-GSK-3 β ; lanes 1 to 3, respectively) were analyzed for PHLPP1 and GSK-3 expression. The numbers shown below the PHLPP1 panel represent the relative expression of PHLPP1 in these cells. (E) The ubiquitination of PHLPP1 is increased in Akt1 knockdown cells. The stable knockdown cells (sh-Con and sh-Akt1; lanes 1 and 2, respectively) were transfected with Myc-Ub, and the cell lysates were immunoprecipitated with the PHLPP1 antibody. The ubiquitination of PHLPP1 was detected using the anti-Myc MAb. The blot was stripped and reprobed using the PHLPP1 antibody to visualize total PHLPP1 in the immunoprecipitates. (F) Diagram showing Akt-mediated negative regulation of PHLPP1 degradation. Our study here demonstrates that PHLPP1 is degraded by β -TrCP-mediated ubiquitination in a phosphorylation-dependent manner. Akt, by inhibiting GSK-3 β activity, negatively controls PHLPP1 degradation.

to as the noncanonical consensus sequence for CK1 (8). In the case of PHLPP1, the sequences surrounding Thr851 and Ser869, **LpTPQDE** and **LpSVEE**, respectively, fit the criteria of the noncanonical consensus sequence. However, if Ser869 is

mutated to Ala, Ser867 may become phosphorylated since it is followed by the two acidic residues (**DpSLSVEE**) as well. Furthermore, the results obtained with the P847 antibody indicated that the level of phosphorylation at Ser847 is deter-

mined by the amount of active GSK-3 β in cells and a priming phosphorylation at Thr851 is required. Since the CK1-mediated phosphorylation is likely to be constitutive, phosphorylation at Ser847 by GSK-3 β provides the switch for controlling the tempo of PHLPP1 degradation.

As the phosphorylation sites are found in PP2C domain of PHLPP1, we analyzed whether the phosphatase activity is affected by the mutations. In vitro phosphatase assays show that the phosphatase activities of GST-PP2C and GST-PP2C/4D are comparable against purified Akt as a substrate. A similar extent of dephosphorylation is achieved with GST-PP2C/4A, although the rate of dephosphorylation is slightly slower than that of the wild-type protein (data not shown). In summary, since the phosphorylated species is rapidly degraded in cells, we believe that the phosphorylation of PHLPP1 does not directly regulate its phosphatase activity. However, the amount of PHLPP1 proteins available in cells can be effectively controlled by signals that regulate the phosphorylation of PHLPP1 in cells.

Akt activity is intrinsically controlled by controlling PHLPP1 expression. Our previous studies have shown that PHLPP1 serves as a negative regulator of Akt by directly dephosphorylating the kinase (14). Here, we found that PHLPP1 is regulated by its substrate Akt as well. It is known that Akt activity is modulated by a negative-feedback loop involving p70S6K-mediated downregulation of IRS proteins (15, 21). Our findings indicate that Akt activity can also be self-regulated by controlling the expression of its phosphatase, PHLPP1. In other words, there is a built-in regulatory mechanism in cells to sense the need for PHLPP1 based on the activation level of Akt. In unstimulated cells, basally phosphorylated PHLPP1 is degraded at a relatively fast rate. Upon stimulation by mitogens, the rate of PHLPP1 degradation slows down due to Akt-mediated inhibition of GSK-3 β . As a result, increased levels of PHLPP1 proteins become available to dephosphorylate Akt in order to prevent Akt hyperactivation. Thus, our results support another feedback model in which the functions of PHLPP1 and Akt are mutually dependent: while the level of PHLPP1 in cells sets the threshold for Akt signaling, the activity of Akt controls the expression of PHLPP1.

Role of β -TrCP-mediated degradation of PHLPP1 in cancer. Ubiquitination is one of the most important posttranslational modifications that plays a pivotal role in maintaining the appropriate level of signaling molecules. β -TrCP-mediated ubiquitination has been linked to cancer by its ability to degrade substrates such as I κ B and β -catenin (12). Interestingly, it has been shown that β -TrCP expression and activity is elevated in colorectal cancer clinical samples with activated Wnt/ β -catenin signaling (29). It is likely that loss of PHLPP expression may occur upon induction of β -TrCP in cancers with elevated Wnt/ β -catenin signaling. Indeed, we find an inverse correlation between PHLPP1 expression and the level of β -TrCP protein in colon cancer cell lines (Fig. 7E). Further studies are needed to determine whether loss of PHLPP1 expression as observed in colon cancer specimens is a result of dysregulation of β -TrCP-mediated ubiquitination pathways. It has been reported recently that PTEN is ubiquitinated by a ubiquitin ligase, NEDD4-1, and increased NEDD4-1 expression correlates with decreased PTEN levels in human bladder cancers (33). Therefore, blocking the ubiquitination process

may present a novel strategy to increase the level of tumor suppressor proteins and enhance their function.

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