Regulation of PTEN Phosphorylation and Stability by a Tumor Suppressor Candidate Protein*

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The tumor suppressor PTEN plays an essential role in regulating signaling pathways involved in cell growth and apoptosis and is inactivated in a wide variety of tumors. In this study, we have identified a protein, referred to as PICT-1 (protein interacting with carboxyl terminus 1), that binds to the C terminus of PTEN and regulates its phosphorylation and turnover. Down-regulation of PICT-1 in MCF7 cells by RNA interference enhances the degradation of PTEN with a concomitant decrease in its phosphorylation. PTEN C-terminal tumor-associated mutants, which are highly susceptible to protein degradation, have lost the ability to bind to PICT-1 along with their reduced phosphorylation, suggesting that their rapid turnover results from impaired binding to PICT-1. Our results identify PICT-1 as a PTEN-interacting protein that promotes the phosphorylation and stability of PTEN. These findings suggest a novel molecular mechanism underlying the turnover of PTEN, which also provides an explanation for the loss of PTEN function due to C-terminal mutations.

The PTEN tumor suppressor antagonizes the actions of phosphoinositide 3-kinase (PI3K)\(^1\) by dephosphorylating the second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP\(_3\)), which is produced by PI3K in response to a variety of stimuli (1–4). Recent genetic studies have revealed that a number of mutations lead the rapid degradation of the mutant PTEN proteins in cells (6), indicating that this region is critical for controlling PTEN turnover. Although PTEN already has been shown to bind to several proteins through the C2 domain or a PDZ-binding motif at the extreme C terminus (7–12), the involvement of these proteins in regulating the turnover of PTEN still remains elusive. Because recent studies have shown that the phosphorylation of specific Ser/Thr residues within the C-terminal region plays an important role in stabilizing PTEN (13–18), it is crucial to unveil the molecular mechanism for the PTEN C-terminal phosphorylation. As a first step toward identifying proteins involved in regulating the phosphorylation and/or turnover of PTEN, we screened for PTEN C-terminal-binding proteins. In this screen we identified a novel PTEN-interacting protein, referred to as PICT-1, encoded by a candidate tumor suppressor gene GLTSCR2 (19). PICT-1 appeared to regulate the phosphorylation and stability of PTEN through its interaction with the C-terminal region. Therefore disruption of the interaction between PICT-1 and PTEN resulted in the rapid degradation of PTEN. Our findings provide insight into the molecular mechanism(s) by which PTEN turnover is controlled.

EXPERIMENTAL PROCEDURES

Plasmids—A cDNA encoding the C-terminal region of human PTEN (residues 338–599) was amplified by PCR from FLAG-PTEN/pCMV5 (20) and subcloned into pAS1-CYH2 (provided by Dr. Stephen J. Elledge) to produce CP/pAS1. Expression vectors for PTEN C-terminal deletion variants (Fig. 1, A and B) were created by cloning corresponding cDNA fragments into pAS1-CYH2 and pGEX-6P-1 (Amersham Biosciences). Tumor-derived C-terminal human PTEN mutants, F341V, V343E, and L345Q, were created by site-directed mutagenesis employing a PCR-based strategy. Human PICT-1 cDNA was amplified by reverse transcription-PCR (RT-PCR) using a 5'-primer that encoded a Myc epitope tag, and the resulting Myc-PICT-1 DNA was subcloned into pOPRSV1-MCS (Stratagene) and pACT2 (Clontech) to create PICT-1/pOPRSV and PICT-1/pACT2, respectively.

Two-Hybrid Screen—To identify proteins that interact with the PTEN C-terminal region, we employed the MATCHMAKER yeast two-hybrid screening system (Clontech). Yeast strain PJ69-2A harboring CT/pAS1 was mated with yeast strain Y187 pre-transformed with a human brain cDNA library according to the manufacturer’s protocol. Mated yeast clones were initially subjected to histidine nutritional selection with 6 mM 3-amino-1,2,4-triazole, and positive clones from the first screening were then further subjected to adenine nutritional selection. Plasmid DNAs were prepared from positive clones and sequenced. A β-galactosidase assay was conducted according to the Clontech Yeast Handbook.

Pull-down Assay—Recombinant PTEN proteins were expressed in Escherichia coli strain JM109 at GST fusion proteins and purified as described previously (20, 21) except that the protease cleavage step was omitted. PICT-1 was translated in vitro from Mac-PICT-1/pORSV plasmid DNA and simultaneously labeled with \(^{35}S\)methionine using the TNT T7 Quick-coupled Transcription/Translation System (Promega) according to the manufacturer’s protocol. After the reaction, GST-fusion PTEN proteins immobilized on glutathione-Sepharose beads (Amersham Biosciences) were added directly to the reaction mixture. After a 12-h incubation at 4 °C, the beads were collected and washed with phosphate-buffered saline, and then bound proteins on beads were analyzed by SDS-PAGE and autoradiography.

Immunoprecipitation—Human breast cancer MCF7 cells (8 × 10\(^6\)) were dispersed by sonication in 1 ml of immunoprecipitation buffer consisting of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 10 mM \(\beta\)-glycerophosphate, 5 mM pyrophosphate, 120 mM NaCl, 1 mM orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride. After

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debris was removed, 7 μg of rabbit IgG or affinity-purified anti-PICT-1 rabbit polyclonal antibody raised against the peptide corresponding to amino acids 37–51 of PICT-1 was added to cleared lysates along with 50 μl of protein A/protein G plus agarose beads (Oncogene). After the samples were incubated for 10 h at 4 °C, the beads were collected by centrifugation and washed with immunoprecipitation buffer, and then proteins remaining on the beads were resolved on SDS-PAGE. The resolved proteins were immunoblotted with anti-PTEN 26H9 antibody.

Immunoblots—Proteins resolved on SDS-PAGE were transferred to a FluoroTrans membrane (Fall), and then immunoblot analyses were performed according to the manufacturer’s protocol. Antibodies used were anti-PTEN (Cascade Bioscience), anti-PTEN 26H9 (Cell Signaling Technology), anti-phospho-PTEN S380 (Cell Signaling Technology), and anti-actin (Sigma). The relative intensity of immunoreactive bands was measured using NIH Image, version 1.62. Typical images from repeated experiments are represented in each figure. All data represent the means ± S.E. from duplicate determinations. Transfections—MCF7 cells (4 × 10^4 cells/well) were cultured in 12-well plates, and transfections were performed using LipofectAMINE 2000 (Invitrogen) or FuGENE 6 (Roche Applied Science) according to the manufacturer’s protocol. Either LipofectAMINE 2000 (4 μl of commercial 1 μg) or FuGENE 6 (3 μl) with 2 μg of DNA was used for each transfection. For co-transfection of siRNA and DNA mixture using 0.2/1 g of small interfering RNA (siRNA) or FuGENE 6 (6.3 μl) with 2 μg of DNA was used for each transfection. For co-transfection of siRNA and DNA (Fig. 3), 4 μl of LipofectAMINE 2000 and 0.2 μg each of siRNA and DNA were used.

Preparation of siRNA—21-Nucleotide RNA duplexes with UU overhangs were used in vitro transcription as described by Yu et al. (22). Target sequences of GLT247 and GLT318 siRNAs correspond to nucleotides 247–267 and 318–338 of GLTSCR2 (GenBank accession No. AF182076) respectively, GFPS control siRNA was described by Yu et al. (22).

RESULTS AND DISCUSSION

Identification of PTEN C-terminal Region-binding Proteins—To identify proteins that interact with the C-terminal region of human PTEN, we performed a two-hybrid screen of a human brain cDNA library using the C-terminal region of PTEN (residues 338–399) as bait. The extreme C terminus of PTEN (residues 400–403), which contains a PDZ-binding motif, was deleted to selectively exclude interactions with PDZ domains. In this screen, we isolated 12 clones of which eight were determined to be false positives and two encoded unknown proteins. Of the two remaining clones, one corresponded to amino acids 10–478 of a protein encoded by a human tumor suppressor candidate gene, GLTSCR2, and the other corresponded to amino acids 673–839 of human Vps16. We have referred to the gene product of GLTSCR2 as PICT-1 because of its ability to bind to the C-terminal region of PTEN. Although both proteins may exert a relevant biological function by binding to PTEN, we have focused herein on the characterization of PICT-1.

Interaction of PICT-1 with PTEN—To confirm our two-hybrid screen results and evaluate the relative contribution of the C-terminal region of PTEN to binding of PICT-1, we first conducted GST pull-down assays using wild-type and deletion mutants of PTEN. The full-length wild-type GST-PTEN fusion protein exhibited significant binding to radiolabeled PICT-1 produced by in vitro translation (Fig. 1A, WT), whereas GST alone did not bind (Fig. 1A, GST). Deletion of the C-terminal segment (338–403) caused a remarkable decrease (by 93%) in the binding of PICT-1 (Fig. 1A, dCT). A construct in which only the C-terminal segment (338–399) of PTEN was fused to GST also exhibited significant binding to PICT-1 (Fig. 1B, CT). However, this fusion protein did not bind PICT-1 as well as the GST-PTEN wild-type bait (32% of wild type). Although this result suggests that the C-terminal segment may not be the sole determinant of PTEN interaction with PICT-1, these data collectively indicate that the C-terminal region of PTEN plays an indispensable role for its interaction with PICT-1. Yeast two-hybrid assay demonstrated that deletion of the 338–348 segment resulted in a complete loss of the binding ability of PTEN to PICT-1 (Fig. 1B), indicating that a critical amino acid residue(s) was located within this 11-amino acid segment. Further, the PDZ-binding motif at the C terminus of PTEN showed no effects on the binding (Fig. 1B).

We also performed a co-immunoprecipitation assay to determine whether an interaction between endogenous PICT-1 and PTEN proteins could be detected in intact cells. For this purpose, we utilized MCF7 cells that contain endogenous PTEN and PICT-1 proteins. As shown in Fig. 1C, the PICT-1 immunoprecipitated from MCF7 cell lysate by the anti-PICT-1 antibody was positive for interaction with endogenous PTEN protein, whereas no PTEN could be detected within the control rabbit IgG immunocomplex. Association of PTEN within the anti-PICT-1 immunocomplex was completely blocked by the corresponding PICT-1 peptide (Fig. 1C, PEP), confirming that endogenous PICT-1 and PTEN proteins can interact in intact cells.

Tumor-associated PTEN C-terminal Mutants Lose the Ability to Bind to PICT-1—The region that appeared to be responsible for the interaction with PICT-1 (Fig. 1, A and B) is nearly identical to a known “hot spot” (341–348) for C-terminal tumor-associated missense mutations (3, 5). Intriguingly, one of these hot spot mutants, L345Q, was reported to exhibit extreme
PTEN mutants were treated with 100 μg/ml cycloheximide (CHX) for 6 h, and then PTEN proteins were detected by immunoblotting. As shown in Fig. 2A, reductions in mutant PTEN levels by 80–90% were observed by 6-h cycloheximide treatment, whereas ~90% of wild-type PTEN remained by the same treatment. Collectively, these findings strongly suggest an essential role for the binding of PICT-1 to PTEN in the stabilization of PTEN protein and provide an explanation for the "loss-of-function" mutations that frequently occur in this region. The C-terminal PTEN mutants, including both deletion and missense mutants that no longer bind efficiently to PICT-1, cannot be protected from degradation and undergo rapid turnover.

PICT-1 Functions as a Regulator of PTEN Turnover—To confirm the role of PICT-1 in regulating PTEN turnover in cells, we next asked whether the down-regulation of PICT-1 directly affects the degradation of wild-type PTEN. For this purpose, we employed an RNA interference method utilizing PICT-1-targeted siRNAs GLT247 and GLT318, which substantially inhibited the ectopic expression of PICT-1 (data not shown). MCF7 cells transfected with FLAG-PTEN/pCMV and either GLT247 or GLT318 were exposed to cycloheximide, and then the level of FLAG-PTEN was assessed by immunoblotting to determine the rate of PTEN degradation (Fig. 3A). In control cells, a 5-h cycloheximide treatment did not significantly affect the level of PTEN, whereas reductions in PTEN levels by 21 and 48% were observed in GLT247- and GLT318-treated cells, respectively (Fig. 3A). Because these findings collectively indicate that PICT-1 plays an essential role in regulating PTEN turnover, we next tested the effect of PICT-1 down-regulation on the steady-state expression level of endogenous PTEN in MCF7 cells. Treatment of MCF7 cells with these siRNAs reduced the level of PTEN by ~40–50%, whereas the control siRNA had no effect (Fig. 3B). It is of note that in this experiment MCF7 cells were cultured longer (48 h) after the siRNA transfection than in the cycloheximide-chase experiment (24 h; see Fig. 3A) to observe a significant effect of PICT-1 down-regulation on endogenous PTEN levels. The residual PTEN protein observed in GLT247- and GLT318-treated samples is likely to be derived from cells into which siRNAs were not effectively delivered and/or because of the relatively long half-life of PTEN protein in MCF7 cells (data not shown, but see Figs. 2C and 3A). The overall reduction in the PTEN expression was coincident with a reduction in the PICT-1 transcript, whereas the level of PTEN and actin mRNAs remained unaffected (Fig. 3C). From these data, we conclude that the reduction in the level of PTEN protein induced by the PICT-1 RNA interference is likely because of changes in the rate of protein degradation.

The involvement of PICT-1 in maintaining PTEN stability also raises the intriguing possibility that PICT-1 loss-of-function mutations may result in the reduction of cellular PTEN in vivo. The GLTSCR2 gene, which encodes PICT-1, is located in a 150-kb minimal common deletion region for human gliomas, and thus it was originally identified as a candidate tumor suppressor gene (19). Down-regulation of PICT-1 expression...
may function to destabilize PTEN and subsequently deregulate PI3K/PIP2-mediated signals, even if no PTEN mutations are present. Our findings suggest a novel tumorigenic pathway that is dependent on the loss of PTEN function but independent of genetic lesions in the PTEN gene. The effect of impaired PICT-1 on PTEN signaling and function could be quite complex. This would be in part because the potential of tissue-specific expression of PICT-1 and PTEN, as well as the phosphorylation of the PTEN C-terminal region, may have multiple effects in addition to promoting PTEN stability (13–18). The significance of PICT-1 as a regulator of PI3K/PIP2-mediated signals is currently under investigation, and further study will be required to demonstrate a biological role for PICT-1.

**PICT-1 Regulates PTEN C-terminal Phosphorylation**—We next looked into the underlying mechanism for the stabilization of PTEN protein by PICT-1. A number of reports have shown that the phosphorylation of Ser/Thr residues within the C-terminal segment of PTEN plays a crucial role in stabilizing the PTEN protein (13–18). However, in the MCF7 cells utilized in this study, phosphorylation of neither Thr-382 nor Thr-383 was likely to contribute significantly to PTEN stabilization, because a phosphorylation-resistant mutant, T382A/T383A, retained its stability in MCF7 cells and its degradation rate was indistinguishable from that of wild-type PTEN (data not shown). Although we cannot rule out a mutually exclusive effect between binding of PICT-1 and phosphorylation at other Ser/Thr residues within the C-terminal region, our findings suggest that Ser-380 is one of the phosphorylation sites that plays a crucial role in the regulation of PTEN turnover.

Our findings also raise the question as to which kinases and phosphatases are involved in the regulation of PTEN turnover. Casein kinase 2 has been implicated in the phosphorylation of the PTEN C-terminal region; however, Ser-380 is a poor match for the casein kinase 2 phosphorylation recognition sequence. It is therefore possible that unidentified kinase(s) may be responsible for this process, and PICT-1 may affect Ser-380 phosphorylation by activating a kinase and/or inhibiting a phosphatase. More detailed study will be required to address these possibilities.

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