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Priority Report

PTEN Acetylation Modulates Its Interaction with PDZ Domain

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Abstract

The *PTEN* tumor suppressor gene is frequently inactivated in human cancer. As a major tumor suppressor, PTEN function must be tightly regulated. Both phosphorylation and membrane association have been reported to regulate PTEN activity. In addition, the COOH terminus of PTEN has a typical PDZ domain-binding motif that interacts with several PDZ domain-containing proteins. In this report, we show that PTEN is acetylated on Lys^{402}, which is in the COOH-terminal PDZ domain-binding motif. We show that CBP plays a major role in PTEN acetylation, whereas the SIRT1 deacetylase is mainly responsible for PTEN deacetylation. Interestingly, Lys^{402} acetylation modulates PTEN interaction with PDZ domain-containing proteins, indicating a potential role of acetylation in regulating PTEN function. [Cancer Res 2008;68(17):6908–12]

Introduction

PTEN, phosphatase and tensin homologue deleted in chromosome 10, is the second most frequently mutated tumor suppressor gene in ~50% of human cancer. The mechanism of PTEN in tumor suppressor function has been clearly defined by its biochemical activity as a lipid phosphatase (1–3). PTEN specifically dephosphorylates the three position of phosphatidylinositols (PI3P, PI3,4P2, and PI3,4,5P3), which are the product of phosphatidylinositol 3-kinase (PI3K). Therefore, PTEN reverses the biological functions of PI3K, which is activated by numerous growth-stimulating signals, such as mitogenic growth factors. Activation of PI3K plays a critical role in the mitogenic and antiapoptotic effects of growth factors.

Although extensive studies have been conducted to show the tumor suppressor function of PTEN, much less is known about PTEN regulation. It is clear that membrane localization of PTEN is important for its biological function as the PTEN substrates are constituents of membrane. The C2 domain of PTEN plays an important role in membrane localization (4–6). Furthermore, the COOH terminus of PTEN contains a typical PSD-95/Dlg/ZO-1 (PDZ) binding motif. Indeed, PTEN has been reported to associate with several PDZ domain-containing proteins and these interactions may play a role in regulating the biological function of PTEN (7–10). However, it is not clear whether and how the association between PTEN and its PDZ domain-containing receptors are regulated.

Protein acetylation is an important posttranslational regulatory mechanism (11). Most of the studies of protein acetylation focus on histone and nuclear transcription regulators (12, 13). Much less is known about lysine acetylation for cytoplasmic proteins. Interestingly, it has been reported that PTEN is acetylated on Lys^{125} and Lys^{128} by PCAF, a histone acetyltransferase (14). In this report, we observed that PTEN is acetylated on Lys^{402}, which is localized in the PDZ domain-binding motif TKV. We identified CBP and SIRT1 as the major acetyltransferase and deacetylase in controlling PTEN acetylation. Our data indicate that acetylation may regulate the interaction between PTEN and PDZ domain-containing proteins.

Materials and Methods

Antibodies and reagents. PTEN and acetyl-lysine antibodies were purchased from Cell Signaling. HA and Myc antibodies were from Covance. Nicotinamide, trichostatin A, and other common chemicals were from Sigma.

Plasmids. Human PTEN cDNA was provided by Dr. Jack E. Dixon (University of California at San Diego, La Jolla, CA). PTEN was subcloned into pRK5-Myc and pRK7-HA vectors to create Myc-PTEN and HA-PTEN expression constructs. Human PCAF was cloned into pCDNA3-FLAG vector to create FLAG-PCAF. HA-SIRT1, SIRT2, FLAG-CBP, and p300 plasmids are provided by Dr. Roland P. Kwok (University of Michigan, Ann Arbor, MI). Human discs large DLG (hDLG) and mouse membrane-associated guanylate kinase inverted-2 (MAGI-2) were provided by Dr. Ben Magolis (University of Michigan) and Dr. Hiromu Sugino (University of Tokushima, Tokushima, Japan), respectively. To create bacterial expression constructs, the fragments containing the second PDZ domain (PDZ2) of hDLG and MAGI-2 were subcloned into pGEX-KG vector.

Small interfering RNA. siGENOME SMARTpool small interfering RNA (siRNA) targeting either SIRT1, SIRT2, CBP, p300, or PCAF was purchased from Dharmacon. Efficiency of siRNA on each target was examined by quantitative reverse transcription-PCR (RT-PCR; Figs. 1*D* and 2C).

RNA isolation and real-time PCR. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen) and subjected to real-time RT-PCR in the presence of Cybergreen (Applied Biosystems). Relative abundance of mRNA was normalized to β-actin mRNA.

Cell culture and transfection. COS7 cells were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin. Transfection was performed using Lipofectamine (Invitrogen).

Immunoprecipitation. Cells were harvested in ice-cold mild lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1% NP40, 50 mmol/L NaF, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin]. The lysates were incubated with HA or Myc antibody for 1 h at 4°C followed by addition of protein G-Sepharose beads and further incubation for overnight. The immunoprecipitates were washed four times with the lysis buffer. The samples were then subjected to SDS-PAGE and analyzed by immunoblotting.

Glutathione *S*-transferase pull-down. The glutathione *S*-transferase (GST) fusion proteins containing the PDZ domain of hDLG or MAGI-2 were expressed in *Escherichia coli* and purified. COS7 cells transfected with HA-PTEN wild-type (WT) or PDZ-binding motif mutants were lysed with mild lysis buffer, and the lysates were incubated with the GST fusion PDZ domain for 1 h at 4°C followed by addition of glutathione-Sepharose beads and further incubation for 2 h. The beads were then washed five times with mild lysis buffer. HA-PTEN proteins in the lysates unbound to the beads were immunoprecipitated with HA antibody. Quantification of immunoblots was done by densitometric analysis using NIH ImageJ software.

PTEN phosphatase assay. HEK293 cells were transfected with HA-PTEN WT or PDZ-binding motif mutants or phosphatase inactive mutant (C124S). HA-PTEN was immunoprecipitated and phosphatase activity was

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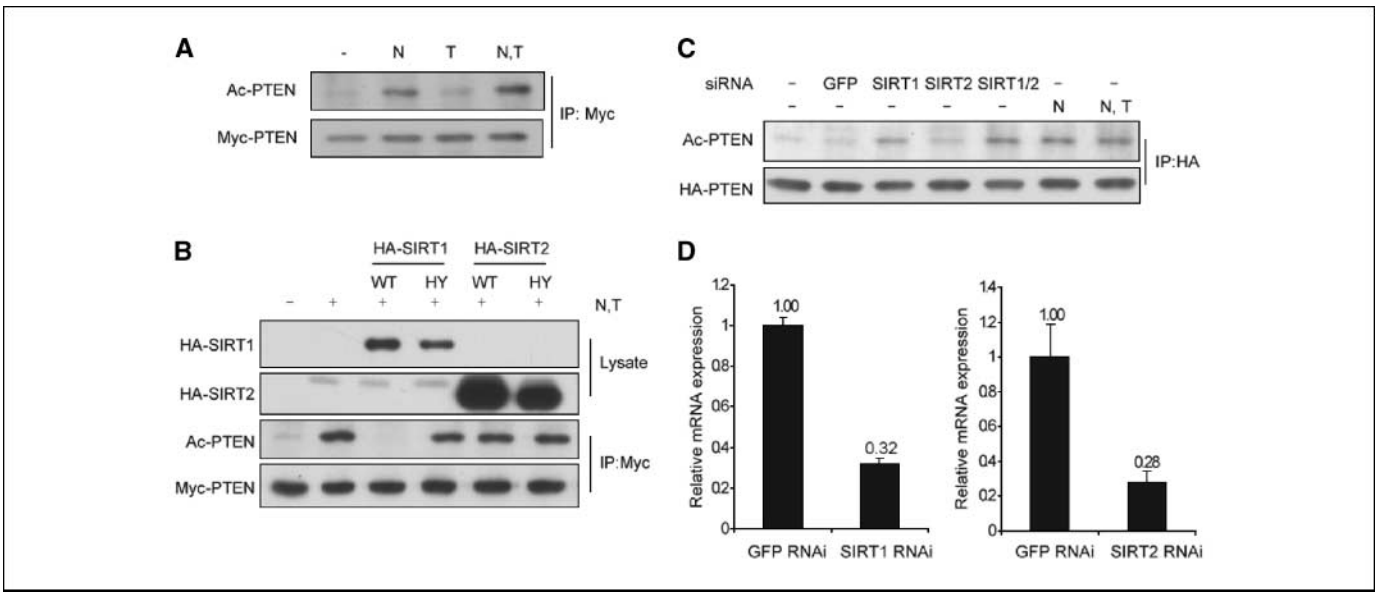

Figure 1. PTEN acetylation is regulated by SIRT1. *A*, nicotinamide increases PTEN acetylation. COS7 cells were transfected with Myc-PTEN. Cells were treated with nicotinamide (*N*), trichostatin A (*T*), or both for 12 h before lysis. Myc-PTEN was immunoprecipitated (*IP*) and the immunoprecipitates were subjected to immunoblotting with anti-Myc or anti–acetylated lysine antibody. *B,* expression of SIRT1 decreases PTEN acetylation. COS7 cells were transfected with Myc-PTEN together with HA-SIRT1 or HA-SIRT2 as indicated. HY denotes the deacetylase inactive mutant of either SIRT1 or SIRT2. Both HA-PTEN and acetylated PTEN levels were analyzed as indicated in *A. C*, knockdown of SIRT1 increases PTEN acetylation. COS7 cells were transfected with HA-PTEN together with siRNA oligos against SIRT1, SIRT2, and GFP (negative control). Treatments with nicotinamide or trichostatin A for 12 h are indicated. HA-PTEN was immunoprecipitated and acetylation was determined. *D*, quantitative RT-PCR shows the efficiency of SIRT1 and SIRT2 knockdown. *RNAi*, RNA interference.

determined using PTEN Malachite Green Assay kit (Upstate Biotechnology) and diC_8-PIP_3 (Echelon) as a substrate.

Results and Discussion

To investigate PTEN acetylation, we tested the effect of nicotinamide and TSA, two deacetylase inhibitors, on PTEN. Initially, we examined PTEN acetylation in HEK293T cells but detected little acetylation (data not shown). We tested several cell lines and found that PTEN acetylation could be detected in COS7 cells (Fig. 1*A*), especially in the presence of nicotinamide treatment, which inhibits the SIRT family of deacetylase. In contrast, treatment with trichostatin A, which inhibits both class I and II of histone deacetylase (HDAC), only had a minor effect on PTEN acetylation. These results indicate that the SIRT family of enzymes plays a major role in PTEN deacetylation in COS7 cells.

To further investigate the involvement of SIRT in PTEN deacetylation, COS7 cells were cotransfected with expression plasmids of PTEN together with SIRT1 or SIRT2 and PTEN acetylation was determined. Coexpression of SIRT1 but not SIRT2 potently decreased PTEN acetylation (Fig. 1*B*). We also tested the catalytically inactive SIRT-HY mutant. As expected, SIRT1-HY mutant did not decrease PTEN acetylation. Furthermore, we performed knockdown experiments and showed that down-regulation of SIRT1 but not SIRT2 caused a significant increase of PTEN acetylation (Fig. 1*C* and *D*). Interestingly, knockdown of both SIRT1 and SIRT2 elevated PTEN acetylation to the level similar to that caused by nicotinamide treatment. These observations are consistent with Fig. 1*A* that nicotinamide but not trichostatin A increased PTEN acetylation. The efficiency of RNA knockdown was confirmed by quantitative RT-PCR for SIRT1 and SIRT2 mRNA. Together, our results strongly indicate that SIRT1 plays a major role in PTEN deacetylation.

It has been reported that PCAF is responsible for acetylation of Lys^{125} and Lys^{128} in PTEN (14). However, the reported study only examined the trichostatin A–sensitive PTEN acetylation, whereas we found that nicotinamide has a more dramatic enhancement on PTEN acetylation in COS7 cells (Fig. 1*A*). We tested the effect of PCAF, p300, and CBP on PTEN acetylation. Coexpression of CBP, but not p300, caused a significant increase of PTEN acetylation (Fig. 2*A*). Surprisingly, PCAF expression only had a minor effect on PTEN acetylation. A possible explanation for the apparent difference between the published data and our observations is the different cell lines used in the two studies. Okumura and colleagues used HEK293T cells, whereas COS7 cells were used in this study.

The function of CBP in PTEN acetylation was further confirmed by knockdown experiments. We found that knockdown of CBP dramatically decreased PTEN acetylation, whereas knockdown of p300 had little effect (Fig. 2*B* and *C*). Interestingly, PCAF knockdown also decreased PTEN acetylation, albeit less dramatic than that caused by CBP knockdown. Collectively, our data show that CBP is the major acetyltransferase for PTEN, whereas PCAF may also contribute to PTEN acetylation.

We next performed experiments to identify the acetylation site in PTEN. Every lysine residues were replaced by arginines individually or in combination with other lysine residue(s). The PTEN mutants were transfected into COS7 cells in the presence of nicotinamide and trichostatin A. PTEN was immunoprecipitated and acetylation was determined. Our data clearly show that arginine substitution at Lys^{402} (K402R) dramatically eliminated PTEN acetylation, whereas mutations of other lysine residues had little effect on PTEN acetylation (Fig. 3). Surprisingly, PTEN K125/128R double mutant was acetylated to the similar extent as the WT protein (Fig. 3*C*), although K125 and K128 were reported as acetylation sites of PTEN by PCAF (14). It is worth noting that the PTEN acetylation observed in our experiments is largely dependent

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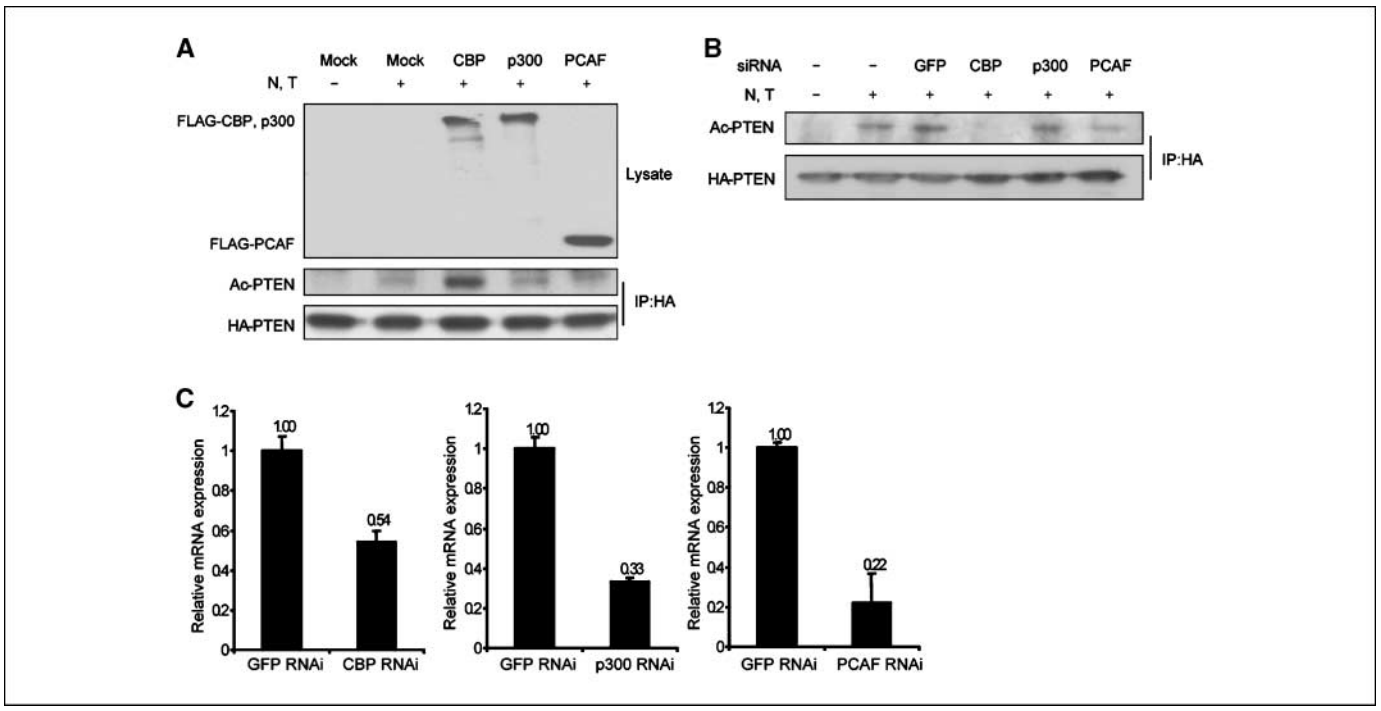

Figure 2. CBP is mainly responsible for PTEN acetylation. *A*, coexpression of CBP increases PTEN acetylation. COS7 cells were transfected with HA-PTEN together with CBP, p300, or PCAF as indicated. Treatments with nicotinamide or trichostatin A for 12 h are indicated. HA-PTEN was immunoprecipitated and the immunoprecipitates were subjected to immunoblotting with anti-HA or anti–acetylated lysine antibody. *B*, knockdown of CBP abolishes PTEN acetylation. COS7 cells were transfected with HA-PTEN together with siRNA oligos targeting CBP, p300, PCAF, or GFP as indicated. Treatment with nicotinamide or trichostatin A was for 12 h. Acetylation of PTEN was determined by methods similar to *A*. *C*, quantitative RT-PCR shows the efficiency of CBP, p300, and PCAF knockdown.

on nicotinamide, whereas Okumura and colleagues only performed experiments with trichostatin A. Furthermore, our experiments were performed in COS7 cells, whereas Okumura and colleagues examined PTEN acetylation in HEK293T cells. Therefore, we speculate that different acetylation sites in PTEN may be regulated differently in different cell types and by different acetyltransferases (CBP versus PCAF) and deacetylases (HDACI/II versus SIRT1).

Interestingly, Lys^{402} is localized in the COOH-terminal PDZ domain-binding motif TKV, which is conserved in vertebrate PTEN (Fig. 4*A*). Previous studies have shown that PTEN interacts with the PDZ domains of MAGI-2, MAGI-3, and hDLG, which are membrane-associated guanylate kinase tumor suppressors (7–9). Our results suggest a model that PTEN acetylation may regulate its interaction with PDZ domain-containing proteins. To test this

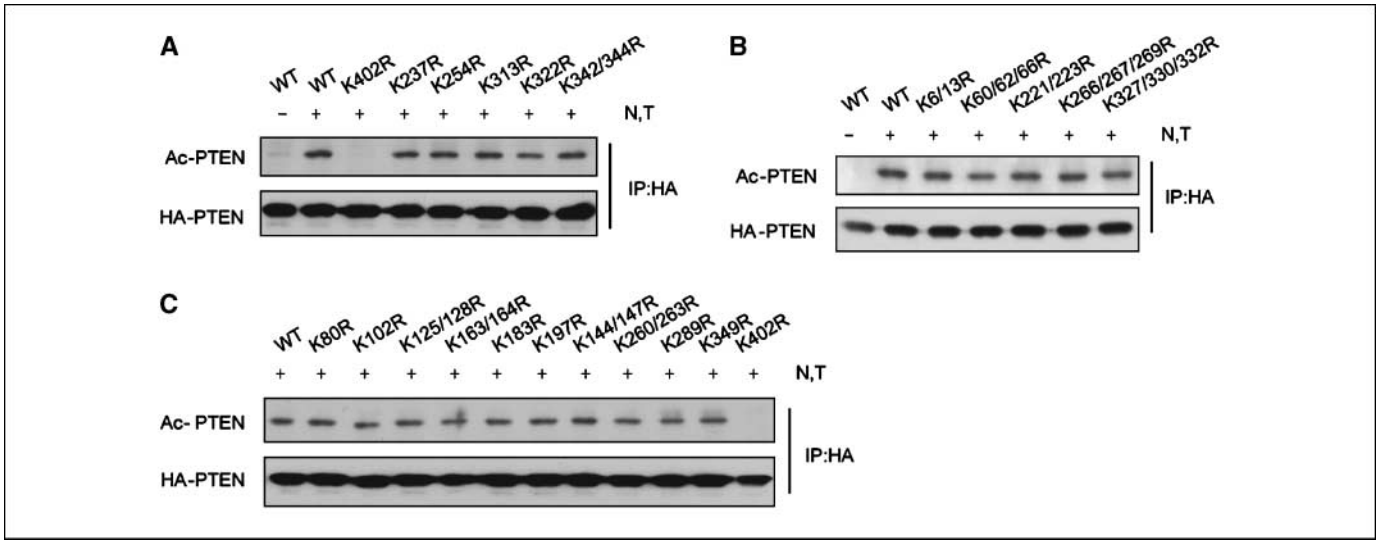

Figure 3. Lys^{402} is the acetylation site in PTEN. Each of 34 lysine residues in PTEN was mutated to arginine individually or in combination with other lysine(s). *A*, arginine mutants on Lys^{237}, Lys^{254}, Lys^{313}, Lys^{322}, Lys^{342}, Lys^{344}, and Lys^{402}. *B*, mutants on K6, K13, K60, K62, K66, K221, K223, K266, K267, K269, K327, K330, and K332. *C*, mutants on K80, K102, K125, K128, K144, K147, K163, K164, K183, K197, K260, K263, K289, K349, and K402. Double and triple lysine mutants are indicated. COS7 cells were transfected with HA-PTEN WT or lysine mutants. The cells were treated with nicotinamide and trichostatin A for 12 h before lysis. HA-PTEN was immunoprecipitated and acetylation of PTEN was determined by anti–acetylated lysine antibody.

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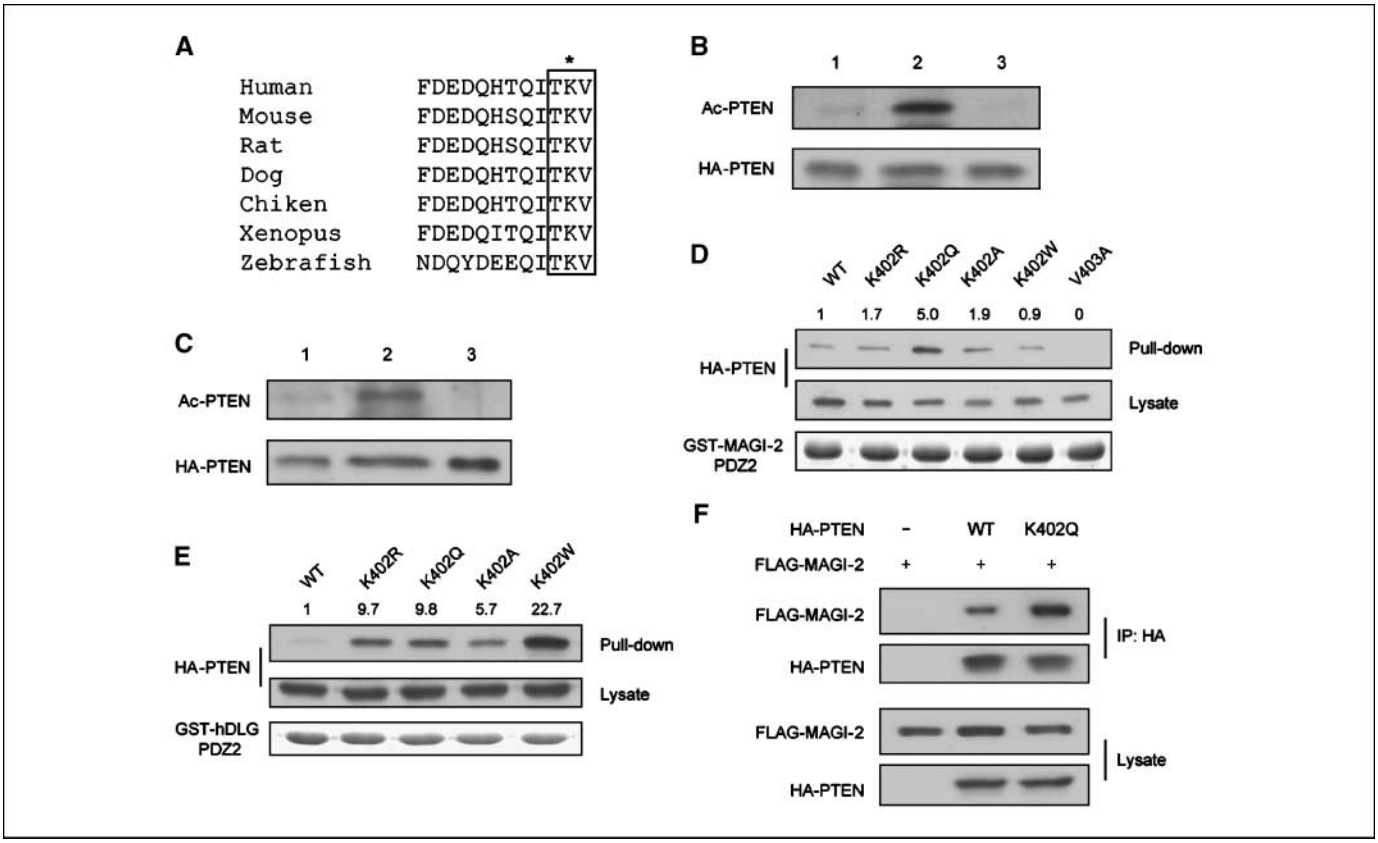

Figure 4. Acetylation of Lys^{402} enhances interaction with PDZ domain. *A*, the PTEN acetylation sites are conserved in vertebrate PTEN. The PDZ domain-binding motifs are boxed. *, acetylation site of human PTEN (K402). *B*, the acetylated PTEN shows a stronger interaction with the hDLG PDZ domain. COS7 cells were transfected with HA-PTEN and treated with nicotinamide and trichostatin A for 12 h before lysis. HA-PTEN was precipitated by HA antibody (*lane 1*) or pull-down by GST-PDZ2 of hDLG (hDLG PDZ2; *lane 2*). *Lane 3*, supernatant after GST pull-down was also precipitated with anti-HA antibody. The precipitates were analyzed for PTEN protein (*bottom*, comparable amounts of HA-PTEN were loaded in three lanes) and acetylation (*top*). *C*, the acetylated PTEN shows a stronger interaction with the MAGI-2 PDZ domain. *Lane 2*, experiments were similar to those in *B* except GST-PDZ2 of MAGI-2 was used in the pull-down. *D*, substitution of K402 by a glutamine in PTEN increases its interaction with MAGI-2 PDZ domain. Various HA-PTEN mutants were expressed in COS7 cells and the cell lysates were incubated with GST-MAGI-2 PDZ2. V403A denotes the substitution of Val^{403} by alanine in PTEN. This mutant cannot be recognized by PDZ domains and is included as a negative control. The protein levels in lysates and GST pull-down were detected by immunoblotting with anti-HA antibody. Quantifications of HA-PTEN proteins in GST pull-down were done by densitometric analysis using NIH ImageJ software and indicated on top of each lane. GST fusion protein levels used in each pull-down were determined by Coomassie blue staining shown in the bottom panel. *E*, mutation of K402 in PTEN increases its interaction with the hDLG PDZ domain. Experiments were similar to those in *D*. *F*, substitution of K402 by a glutamine in PTEN increases its interaction with MAGI-2 *in vivo*. COS7 cells were transfected with HA-PTEN WT or K402Q mutant together with FLAG-MAGI-2. HA-PTEN in the lysates was precipitated with anti-HA antibody and coprecipitated FLAG-MAGI-2 was determined by immunoblotting.

possibility, HA-PTEN–containing cell lysates were immunoprecipitated using anti-HA antibody or subjected to an *in vitro* pull-down using GST-PDZ domain of hDLG or MAGI-2. The HA-PTEN protein in the supernatant after GST-PDZ pull-down was also precipitated with HA antibody. The relative levels of acetylation were compared. We found that the acetylated HA-PTEN was preferentially enriched in complex with GST fusion of PDZ2 of hDLG (Fig. 4*B*, compare *lane 1* and *lane 2*). Furthermore, the HA-PTEN in the supernatant after GST-hDLG PDZ2 pull-down showed a much lower level of acetylation (Fig. 4*B, lane 3*). We also performed similar *in vitro* pull-down experiments with the PDZ2 of MAGI-2. Consistent with the results with hDLG, GST-MAGI-2 preferentially bound the acetylated PTEN (Fig. 4*C, lane 2*). These results show that acetylation of PTEN may enhance its interaction with PDZ domains.

The effect of PTEN acetylation on phosphatase activity was investigated. We mutated the acetylation of Lys^{402} in several different residues in hoping to create mutants that may be acetylation mimetic or abolishing acetylation. We found that substitution of K402 by glutamine, arginine, and tryptophan had little effect on PTEN phosphatase activity, whereas mutation of the active site Cys^{124} completely abolished phosphatase activity (data not shown). These results indicate that acetylation of PTEN K402 may not directly affect the phosphatase activity.

The ability of PTEN K402 mutants to interact with PDZ domain was examined. We found that only the K402Q mutant significantly increased its interaction with GST-MAGI-2 PDZ2 (Fig. 4*D*), suggesting that glutamine substitution of Lys^{402} may mimic the effect of acetylation. The specificity of this interaction was confirmed by the PTEN mutant V403A, in which the Val^{403} was replaced by alanine. The PTEN V403A mutant indeed abolished the interaction with GST-MAGI-2 PDZ2 (Fig. 4*D*), consistent with previous observation (15). Similar *in vitro* binding assay was performed with GST-hDLG PDZ2. The PTEN K402Q mutant significantly increased its interaction with the hDLG PDZ domain 2 (Fig. 4*E*). Interestingly, the other PTEN mutants, including K402R and K402W, also significantly increased their interactions with GST-hDLG PDZ2. Finally, we performed coimmunoprecipitation experiment of PTEN and MAGI-2. The PTEN K402Q mutant interacted with full-length MAGI-2 more strongly than the WT PTEN, indicating that acetylation of PTEN increases binding to

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PDZ domain in the cell (Fig. 4*F*). These results further support a possible role of K402 acetylation in modulating PTEN interaction with PDZ domains and also indicate that the recognition of PTEN COOH terminus by MAGI-2 PDZ and hDLG PDZ is not identical.

To test the effect of acetylation on PTEN localization, we performed immunofluorescence in cells treated with nicotinamide and trichostatin A. We found that these two inhibitors had no effect on PTEN localization (data not shown). Similar results were obtained by subcellular fractionation experiments. This could be due to the possibility that only a small fraction of PTEN is acetylated. In addition, we examined the localization of the acetylation mimetic PTEN K402Q and did not detect a difference in localization (data not shown). Therefore, the functional significance of acetylation in PTEN localization is not clear. It is possible that acetylation does not affect PTEN localization. Alternatively, acetylation may contribute to the localization of a small fraction of intracellular PTEN by modulating its interaction with PDZ domain-containing proteins. Third, it is also possible that multiple signals control PTEN localization, such as the C2 domain and acetylation, whereas acetylation alone is not sufficient to dictate PTEN localization.

In this study, we show that Lys^{402} in the PDZ domain-binding motif of PTEN is acetylated. This acetylation likely modulates PTEN functions, particularly on its interaction with PDZ domain-containing proteins. Our data showed that acetylation enhances the interaction between PTEN and PDZ domains. Acetylation will produce two obvious effects: one to eliminate the positive charge and the other to alter the size of the side chain. Substitution of lysine by glutamine in PTEN results in a significant increase of interaction with the PDZ domains of both hDLG and MAGI-2, suggesting that glutamine may serve as acetylated lysine mimetic. However, substitution of lysine by other residues, such as arginine and tryptophan, also increases interaction with the PDZ2 of hDLG, indicating that masking positive charge is not the only factor contributing to the regulation of lysine acetylation in PDZ binding. It is possible that acetylation not only increases the affinity of PTEN to PDZ domain-containing proteins but also affects PTEN-interacting partner selectivity. Consistent with this notion, the PDZ domain of MAGI-2 shows a higher selectivity for residues at position 402 in PTEN than the PDZ domain of hDLG.

Okumura and colleagues (14) identified Lys^{125} and Lys^{128} as the acetylation sites in PTEN. We performed our PTEN acetylation experiments in the presence of nicotinamide because nicotinamide treatment caused a dramatic increase of PTEN acetylation. However, Okumura and colleagues performed their experiments in the absence of nicotinamide. In addition, our experiments were performed in COS7 cells, whereas Okumura and colleagues did the experiments in HEK293T cells. Therefore, a possible explanation is that CBP and SIRT1 are mainly responsible for PTEN K402 acetylation in COS7 cells, whereas PCAF and HDACI/II are mainly responsible for PTEN K125/K128 acetylation in HEK293T cells.

We show that SIRT1 plays a key role in PTEN deacetylation based on the following evidence. Nicotinamide treatment significantly increased PTEN acetylation. Expression of SIRT1 decreased PTEN acetylation. Furthermore, knockdown of SIRT1 increased PTEN acetylation. SIRT1 expression is elevated in human prostate cancer and inhibition of SIRT1 has been reported to suppress tumor growth and induce apoptosis (16–18). The regulation of PTEN acetylation by SIRT may provide a potential mechanism for SIRT1 in tumorigenesis.

Our data indicate that CBP plays a major role in PTEN acetylation based on both coexpression and siRNA-mediated knockdown. It is particularly intriguing that CBP and PCAF exist in the same complex. An interesting model is that the two different histone acetyltransferase activities in the CBP/PCAF complex acetylate PTEN on different lysine residues. One may speculate that acetylation of Lys^{125} and Lys^{128} by PCAF inhibits PTEN lipid phosphatase activity, whereas acetylation of Lys^{402} by CBP modulates PTEN interaction with PDZ domain-containing proteins. Therefore, multiple acetylations controlled by different acetyltransferases and deacetylases may affect the PTEN biological functions by multiple mechanisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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