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Advances in Brief

Regulation of PTEN Binding to MAGI-2 by Two Putative Phosphorylation Sites at Threonine 382 and 383[1]

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Abstract

We have reported previously that the PTEN COOH-terminal 33 amino acids play a role in the maintenance of PTEN protein stability (Tolkacheva and Chan, Oncogene, *19:* 680–689, 2000). By site-directed mutagenesis, we identified two threonine residues within this COOH-terminal region at codon 382 and 383 that may be targets for phosphorylation events. Interestingly, PTEN mutants rendered phosphorylation-incompetent at these two sites, T382A/T383A, and were found to have drastically reduced expression in cultured cells. The enhanced degradation of PTEN was most likely mediated by the proteosome-dependent pathway, we have evidence that PTEN was polyubiquitinated. More interestingly, the non-phosphorylated forms of PTEN displayed significantly greater binding affinity than the wild-type protein to a previously identified PTEN interacting partner, MAGI-2/ARIP1. On the basis of all these data, we propose that PTEN recruitment to the cell-cell junction may be regulated through the phosphorylation of its COOH terminus.

Introduction

PTEN is a *bona fide* human tumor suppressor gene which encodes a protein with phosphatase activity toward PI^3 (1–3). The frequent inactivation of PTEN in various human tumors provides strong evidence that uncontrolled PI turnover plays a role in tumor formation (4). Indeed, components of the PI-dependent signaling cascade, such as growth factor receptors, Ras, Akt, and PI3-K have been shown to be either constitutively activated or overexpressed in human cancer (5). The ectopic expression of *PTEN* in various tumor cell lines has been shown to exert inhibitory effects on several known biological actions of PI3-K, such as proliferation, invasion, and cell survival (6).

Biochemically, PTEN mediates the dephosphorylation of the D3-phosphate of PIP3 (7–9). On the basis of the primary amino acid sequence and the recently resolved crystal structure (10), PTEN can be generally divided into two functional regions. In the NH_2-terminal half of the molecule resides the phosphatase and Tensin-homology domains which binds and catalyzes the dephosphorylation of phospholipid substrates. The COOH-terminal half of PTEN is most likely regulatory in nature and is composed of three sub-domains: (*a*) a C2 domain that has been implicated in phospholipid-binding; (*b*) two tandem PEST domains that are believed to play a role in controlling protein stability; and (*c*) a PDZ-binding domain which may play a role in protein-protein interaction.

Insights into the regulation of PTEN phosphatase activity were provided by the identification of binding proteins that interact with the COOH-terminal PDZ-binding consensus sequence of PTEN. By screening yeast two-hybrid libraries with PTEN as bait, three separate groups have identified members of the MAGUK family, hdlg, hMAST205, ARIP1/AIP1/MAGI-2 (hereafter referred to as MAGI-2), and MAGI-3, to interact directly with PTEN (11–13). Common to all these proteins are the possession of five to six PDZ domains, two WW domains and a guanylate kinase-like domain. Immunocytochemical analysis of MAGUK proteins has shown them to be localized to cell-cell junctions. Also, expression of MAGI-2 enhances the ability of PTEN to down-regulate the PI3-K-dependent signaling cascade (12). These results lead to the speculation that MAGUK proteins, by recruiting PTEN to the plasma membrane, promote the dephosphorylation of PIP3 in that cellular compartment. However, it is unclear how the interaction between PTEN and MAGUK family proteins is being regulated.

In this study, we report the identification of two potential phosphorylation sites in the PTEN COOH terminus that may play a role in controlling the binding affinity to the second PDZ domain (PDZ2) of MAGI-2. Our results provide a model whereby PTEN localization and activity in a cell could be regulated through phosphorylation.

Materials and Methods

Cell Culture. The NIH3T3 cell line was maintained in DMEM supplemented with 10% calf serum. The 293T and PC3 cell lines were maintained in DMEM supplemented with 10% FCS. For transfecting 293T cells, the standard calcium phosphate precipitation method was used. For gene transfer in PC3 cells, $\sim 10^6$ cells on a 60-mm culture dish were lipofected with 1–2 μg of DNA using 8 μl of Lipofectamine (Life Technologies, Inc., Rockville, MD) in 1.4 ml of serum- and antibiotic-free DMEM. After 5 h of incubation at 37°C, cells were then rinsed once and replaced with 3 ml of growth medium. Most biological assays were performed within 36–48 h after transfection.

Plasmids. PTEN-WT, PTEN-AA, and various PTEN COOH-terminal truncated cDNAs have been described previously (14). For the generation of PTEN T382A, T383A, T382/3A, T382E, T383E, and T382/3E phosphorylation site mutants, standard PCR-based site-directed mutagenesis was used. The authenticity of all constructs was confirmed by nucleotide sequencing analysis. All PTEN mutant cDNAs were restriction-digested with *Bam*HI and *Eco*RI enzymes and fused in-frame in the NH_2 terminus to an AU5-epitope tag present in the expression vector, *pCEFL KZ AU5*. The HA-tagged ubiquitin expression plasmid, pCMV-HA-Ub, was a gift from Dr. Zeev Ronai (Mt. Sinai School of Medicine, New York, NY) and was described previously (15). A Flag-tagged MAGI-2 (also referred to as AIP1 or ARIP1) expression construct (pSαFlagN-MAGI-2) was generated by fusing a ~3.5 kb *Eco*RI-*Eco*RV cDNA fragment to a Flag-epitope containing expression vector, pSα (16). For the creation of a GST-fusion protein of the PDZ2 domain of MAGI-2, two restriction enzyme-tagged primers were used to amplify a 285-bp region corresponding to the codon residues from 594 to 688 of MAGI-2. The

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[3] The abbreviations used are: PI, phosphatidylinositide; PI3-K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3,4,5 triphosphate; MAGUK, membrane-associated guanylate kinase; WT, wild type; AA, catalytic-inactive (phosphatase-defective); GST, glutathione *S*-transferase; α-p-Thr, anti-phospho-threonine antibody; HA-Ub, hemagglutinin-tagged ubiquitin.

amplified product was restriction digested with *Bgl*II and *Eco*RI and subcloned into the *Bam*HI and *Eco*RI sites of the *pGEX-KG* expression vector.

Antibodies. The anti-HA (12CA5) monoclonal antibody was derived from the Monoclonal Core Facility of the Mount Sinai School of Medicine. The anti-PTEN mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), the anti-actin mouse monoclonal antibody (Santa Cruz Biotechnology), the anti-AU5 monoclonal antibody (Covance, Richmond, CA), the anti-Flag monoclonal antibody (Sigma Chemical Co., St. Louis, MO), the anti-phos-threonine rabbit polyclonal antibody (Sigma Chemical Co. and Zymed Labs, Inc., South San Francisco, CA) were purchased from commercial sources. The anti-PTEN rabbit polyclonal antibody was generated by immunizing rabbit with a fusion protein of GST and the COOH-terminal 216 amino acids of PTEN.

PTEN-MAGI-2 Binding Assay. For testing binding between MAGI-2 and PTEN mutants, 5 μg of pSαFlagN-MAGI-2 was transiently transfected into 5×10^6 293T cells per 100-mm culture dish. Approximately 36 h after transfection, cells were solubilized with 700 μl of standard radioimmunoprecipitation assay buffer, and Flag-tagged MAGI-2 was affinity purified using 20 μg of anti-Flag antibody coupled to 150 μl of γ-bind G-Sepharose beads (Pharmacia, Piscataway, NJ). To generate PTEN substrates for the binding experiments, 2×10^6 293T cells in a 100-mm dish were transfected with 5–10 μg of various PTEN expression constructs. Approximately 24 h after transfection, cells were solubilized in 700 μl of a lysis buffer composed of 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA (pH 8.0), 1 mM Na_3VO_4, 1 mM $Na_3P_3O_4$, 10 mM NaF, 2 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, and 1 mM aprotinin. For a typical binding assay, 30 μl of the Flag-MAGI-2-containing beads was incubated with ~1 mg of total cell extract expressing similar levels of individual PTEN mutants. Binding was carried out by turning the reactions at 4°C for 2 h and then washing three times with lysis buffer. Bound PTEN proteins were eluted by boiling in 60 μl of a Laemmli sample buffer. An equivalent amount of each binding reaction was loaded on a 12.5% SDS-PAGE gel with subsequent standard Western blot analysis using a rabbit anti-PTEN polyclonal antibody. To test the ability of the PDZ2 domain of MAGI-2 to bind to different PTEN mutants, the fusion protein GST-MAGI2-PDZ2 was synthesized and affinity purified as described previously (17). For a typical binding experiment, we used 25 μl of glutathione beads coupled with ~30 μg of GST-MAGI2-PDZ2 fusion protein. Binding reactions with various PTEN mutants and subsequent washing conditions were essentially the same as described above.

Results

Identification of Two Potential Phosphorylation Sites in PTEN Protein at Codons 382 and 383. We have demonstrated previously that the PTEN COOH terminus plays a role in conferring protein stability (14). As has been well-documented in the case of p53 tumor suppressor protein, phosphorylation plays a critical role in the regulation of proteosome-mediated protein degradation (18). We speculated that PTEN may also use a similar mechanism, and an attempt was made to identify potential phosphorylation sites within the COOH-terminal 33 amino acids of PTEN. Using a panel of commercially derived phospho-specific antibodies, the phosphorylation states of PTEN protein overexpressed in cultured cells were investigated. We failed to detect both serine and tyrosine phosphorylation using these immunological reagents (data not shown). However, a positive signal was registered when an α-p-Thr was used (Fig. 1). Similar results were also obtained when a different commercially available antibody was tested under similar experimental conditions. However, this p-Thr signal was not detected using bacterially generated PTEN proteins, suggesting that the immunoreactivity observed was most likely attributable to *bona fide* phosphorylation events in cultured cells (data not shown).

To identify the threonine residues in question, we performed similar transient transfection experiments using various COOH-terminal truncated PTEN mutants as described previously (14). As shown in Fig. 1, deleting the 4-amino acid PDZ-binding domain from the PTEN COOH-terminus (*C*Δ*4*) did not significantly affect

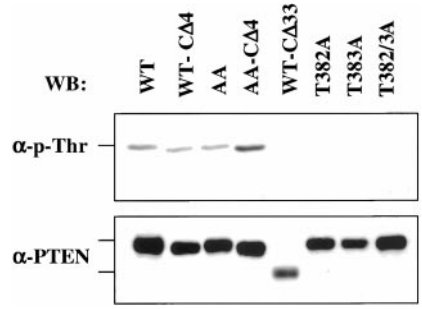

Fig. 1. PTEN is phosphorylated on threonine residues. Different AU5-epitope-tagged PTEN constructs representing the WT (*WT*), phosphatase-defective (*AA*), COOH-terminal deletion mutants lacking 4 (*C*Δ*4*) or 33 (*C*Δ*33*) amino acids, and three phosphorylation site mutants (*T382A*, *T383A*, and *T382/3A*) were ectopically expressed in 293T cells. PTEN proteins were immunoprecipitated from total cell extracts with an anti-AU5 monoclonal antibody and resolved on an SDS 12.5% polyacrylamide gel. Western blot analysis was carried out using an α-p-Thr (*top*) or an anti-PTEN rabbit polyclonal antibody (*bottom*).

the magnitude of α-p-Thr reactivity, irrespective of whether PTEN was in its WT or AA states. However, deleting 33 amino acids of PTEN COOH-terminus (*C*Δ*33*) abrogated all of the p-Thr signal. The fact that there were only two threonine residues within this region at codon 382 and 383 (Thr-382 and Thr-383) led us to believe that they may serve as targets for phosphorylation. To test this possibility, we rendered these two threonine residues either singly (T382A and T383A) or doubly (T382/3A) defective for phosphorylation by substituting them with alanine residues. Indeed, α-p-Thr failed to detect a significant signal in all three PTEN mutants (Fig. 1). The fact that mutating just one of the two threonines resulted in a nearly complete abrogation of the cross-reactivity suggests that α-p-Thr showed a higher affinity toward the doubly phosphorylated PTEN molecules. Alternatively, phosphorylation at these threonine residues may show a certain mutual codependency.

PTEN Phosphorylation Site Mutants Affect PTEN Protein Stability. To address whether phosphorylation events at Thr-382 and Thr-383 could alter PTEN protein stability, transient transfection experiments were performed in a PTEN-null prostate cancer cell line, PC3. As shown in Fig. 2*A*, when an equal amount of expression vector was transfected, there was a ~10–20 fold reduction in the levels of T382A and T383A mutants when compared with the wild-type protein 24 h after transfection. The double phosphorylation-defective mutant, T382/3A, also was less expressed but consistently displayed a higher expression level than the single-site mutants. All three phosphorylation-defective mutants were almost undetectable 48 h after transfection. In contrast, the three phosphomimic mutants (T382E, T383E, and T382/3E) still retained considerable levels of protein expression at the 48 h time point.

To explore whether the observed decreases in the expression levels of PTEN phosphorylation site mutants were attributable to a proteosome-mediated protein degradation pathway, we performed similar transient transfection expression analysis in the presence or absence of the proteosome inhibitor, ALLN. Transfected cells were treated with either control solvent, DMSO, or 40 μM of ALLN for 2 and 4 h. Whereas the levels of PTEN-WT were not significantly affected by ALLN, all three phosphorylation site mutants displayed a 2–5 fold increases in protein expression with time (Fig. 2*B*).

One of the critical steps in the proteosome degradation pathway is the formation of a ubiquitin-protein conjugate (19). The covalent addition of multiple ubiquitin molecules to the target protein is prerequisite for its degradation by the 26S proteosome. To test whether PTEN was ubiquitinated, we cotransfected various forms

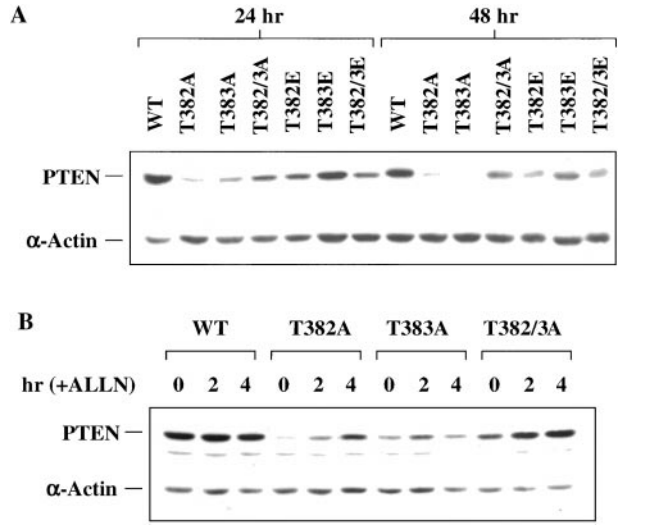

Fig. 2. Non-phosphorylated forms of PTEN are unstable. *A*, equal amounts of indicated plasmids were transfected into a PTEN-null PC3 cell line. Cultures were solubilized at 24 or 48 h, and equal amount of cell extracts were resolved on a SDS 12.5% polyacrylamide gel. *B*, similar transfection reactions were performed, and 24 h after transfection, cultures were exposed to ALLN for 0, 2, and 4 h before solubilization. The expression levels of various PTEN isoforms were monitored with an anti-PTEN antibody, and protein loading was normalized with an anti-actin antibody.

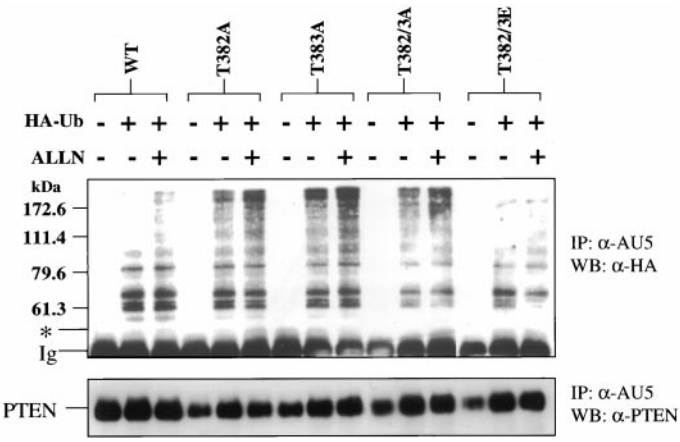

Fig. 3. PTEN is polyubiquitinated *in vivo*. Transient transfection was carried out in 293T cells by coexpressing the indicated PTEN expression plasmids with (+) or without (−) an HA-tagged ubiquitin (*HA-Ub*) construct. Approximately 48 h after transfection, cultures were treated with (+) or without (−) ALLN for 4 h before solubilization. PTEN proteins were immunoprecipitated (*Ip*) from total cell extracts with an anti-AU5 monoclonal antibody and Western blot (*WB*) analysis was carried out to detect the polyubiquitinated chains using an anti-HA antibody (*top*). The expression levels of PTEN were monitored with an anti-PTEN rabbit polyclonal antibody (*bottom*). *, position of PTEN. The cross-reactive mouse immunoglobulin heavy chain is indicated.

of PTEN with a HA-Ub expression construct in 293T cells. Transfected cultures were either treated with or without ALLN for 4 h before cell lysis. Next, PTEN proteins were immunoprecipitated from total cell extracts, and covalently linked HA-Ub was detected by immunoblot with an anti-HA antibody. As expected, the characteristic multimeric banding patterns of ubiquitinated-protein conjugates were clearly demonstrated (Fig. 3). The observed result was specific, because a similar HA-Ub ladder was not detected in cells transfected with a vector control (data not shown). In addition, the more rapidly degraded T382A and T383A mutants displayed an apparently greater intensity of the HA-Ub conjugation events than either the WT or the phosphomimic mutant T382/3E. Furthermore, in all cases, the addition of ALLN augmented the magnitude of polyubiquitination significantly. On the basis of all these results, we conclude that dephosphorylation at Thr-382 and Thr-383 leads to the increases in PTEN degradation potentially mediated by the ubiquitin-proteosome pathway.

PTEN Phosphorylation Site Mutants Bind MAGI-2 PDZ2 Domain with Elevated Affinity. Previous experiments have suggested that, by recruiting PTEN to the cell-cell junction, the MAGUK family of proteins, such as MAGI-2, enhances PTEN tumor-suppressor function (12). The proximity of Thr-382 and -383 to the PDZ-binding domain led us to test whether PTEN binding to MAGI-2 could be regulated by phosphorylation of these residues. For this, we separately overexpressed a Flag-tagged MAGI-2 and various PTEN mutants in 293T cells. Binding reactions were carried out by adding cell extracts expressing similar amounts of various PTEN mutants to an affinity column with bound Flag-MAGI-2. Interestingly, all three phosphorylation-defective mutants displayed a strikingly greater binding capacity of 15–30-fold over their WT counterpart (Fig. 4*A*). As expected, the PTEN mutant lacking the 4-amino acid PDZ-binding domain (*PTEN-CΔ4*) failed to interact with Flag-MAGI-2.

Because it has been reported that PTEN binds with the highest affinity to the second PDZ domain of MAGI-2 (MAGI2-PDZ2; Ref. 12), we sought to test whether different PTEN phosphorylation site mutants also exhibited differential binding to this domain. For this, we developed an *in vitro* binding assay using a recombinant GST-MAGI2-PDZ2 fusion protein immobilized on glutathione beads. We performed extensive control experiments, showing that PTEN expressed in cells did not bind either to control glutathione beads or to GST alone (data not shown). Also, as would have been expected, GST-MAGI2-PDZ2 failed to bind to the PTEN-Δ4 mutant. More importantly, we were able to affinity purify endogenous PTEN from

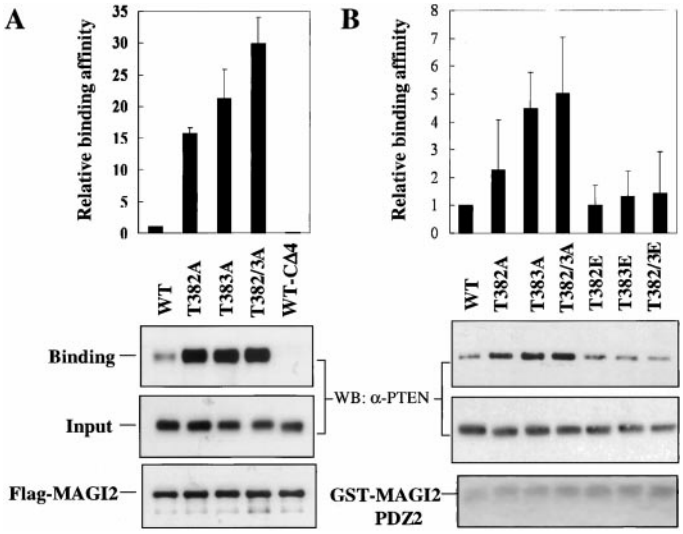

Fig. 4. Non-phosphorylated forms of PTEN bind MAGI-2 with higher affinity. *A*, Flag epitope-tagged MAGI-2 and indicated PTEN expression plasmids were separately expressed in 293T cells. Binding reactions were carried out as described in "Materials and Methods." The levels of PTEN bound to Flag-MAGI-2 (*Binding*) and the relative levels of PTEN added to the binding reactions (*Input*) were monitored using an anti-PTEN antibody. The amount of Flag-MAGI-2 immobilized onto the affinity columns was detected using an anti-Flag monoclonal antibody (*bottom*). *B*, similar binding assays were performed, but GST-MAGI2-PDZ2 fusion proteins immobilized on glutathione beads were used instead. Western blot (*WB*) analysis was carried out as described in *A*, and the amount of GST-MAGI2-PDZ2 fusion proteins was visualized by Coomassie Blue stain (*bottom*). The extent of binding was quantified by an imaging densitometer, and data (*top*) are expressed as *relative binding affinity*. *Bars*, SDs derived from a representative experiment performed in triplicate, which has been repeated at least two times with similar results obtained.

total cell extracts derived from NIH3T3 fibroblasts, attesting to its suitability as an affinity probe.[4]

Next, we performed similar binding assays using a panel of PTEN phosphorylation site mutants. We observed that the three phosphorylation-defective mutants of PTEN displayed a 2–5-fold greater relative binding to GST-MAGI2-PDZ2 than the WT protein (Fig. 4*B*). Furthermore, the double mutant, T382/3A, showed a consistently higher binding capacity in multiple experiments. In contrast, all of the three phosphomimic mutants (T382E, T383E, and T382/3E) bound to GST-MAGI2-PDZ2 at a level similar to that of the PTEN-WT protein. We conclude from these experiments that dephosphorylation of PTEN at Thr-382 and -383 increases interaction with MAGI-2 most likely through the PDZ2 domain. Also, it is possible that the recruitment of PTEN to the cell-cell junction by MAGI-2 is promoted by dephosphorylation events.

Discussions

In this study, we report the identification of two threonine phosphorylation sites in the PTEN COOH terminus that regulate both protein stability and its interaction with MAGI-2. Our data are very similar to two recent reports by Vazquez *et. al*. (20) and Torres *et. al*. (21), in which both groups have identified the same two residues as being phosphorylated *in vivo*. Furthermore, these two studies also reported additional phosphorylation sites at Ser-370, Ser-380, and Ser-385. Our inability to detect these serine phosphorylation sites could be explained by the limitation of the commercially available anti-phosphoserine antibodies. However, analysis of the individual phosphoacceptor site mutants indicates that only Ser-380, Thr-382, and Thr-383 are more critical in controlling PTEN protein stability and biological activities (20, 21).

It is not known, however, the relative phosphorylation states at individual Ser/Thr residues in PTEN-WT protein under different physiological conditions. Also, we have attempted but failed to demonstrate that the phosphorylation events could be modified under different cell culture conditions. These include mitogen stimulation, adherent *verses* nonadherent growth, and exposure to different stress conditions. On the basis of comparisons of the relative binding capacity between WT and phosphorylation-site mutants to MAGI-2, we estimate that ~95% of PTEN in a cell undergo phosphorylation at one or both of the residues. Therefore, we speculate that the majority of PTEN *in vivo* are phosphorylated, cytoplasmically localized, relatively stable, but functionally inert. Intriguingly, we consistently observe the double mutant (T382/3A) to be more stable than the two single phosphorylation site mutants (T382A and T383A; Fig. 2) in our experiments. Coincidentally, the double mutant also displays a greater binding affinity toward MAGI-2 than the single-site mutants (Fig. 4). Therefore, it is tempting to speculate that PTEN binding to MAGI-2 may have some stabilization effect that could be crucial for its phosphatase function at the cell-cell junction.

Data generated in the present study as well as others (20, 21) have indicated strongly that phosphorylation of the COOH-terminal PEST domain of PTEN alters its protein stability. Furthermore, we have extended these findings by providing the first evidence that PTEN may undergo ubiquitination. It appears paradoxical that proteosome inhibitor treatment did not significantly alter the PTEN-WT protein level. This can be explained by our earlier estimation that over 95% of PTEN in a cell exists in a relatively stable phosphorylated form with a reported half-life of ~4 h (20–22). Thus, the treatment with ALLN would not be expected to elevate further the level of total PTEN protein. However, the small pool of hypophosphorylated and unstable PTEN is revealed in the form of ubiquitinated species when PTEN-WT is overexpressed in 293T cells (Fig. 3). Whether PTEN undergoes ubiquitination under physiological conditions is unclear at present.

It is predicted that the COOH-terminal 50-amino acid region of PTEN, where the Ser/Thr sites reside, is relatively unstructured (10). The flexible nature of this motif may well play a crucial role in the regulation of PTEN tumor suppressor functions through phosphorylation. For example, a hypophosphorylated, less-structured PTEN COOH terminus may relieve some of the steric hindrance that would normally prevent the substrate PIP3 from occupying the catalytic pocket. Alternatively, a flexible PTEN COOH-terminus may render the PDZ-binding domain more exposed, making it available for MAGI-2 binding. Our data tend to support the latter hypothesis, because the T382A/T383A mutants display a 15–30-fold greater binding capacity to MAGI-2 than their WT counterpart. Our findings, therefore, could partly explain why PTEN phosphorylation site mutants described by Vazquez *et. al*. (20) are relatively more active than WT protein in suppressing FKHR transcriptional activity and inducing G_1 arrest (20).

On the basis of the results derived from the present study as well as other published data, it is possible then to postulate an activation model for PTEN. In response to an as yet unknown stimulus, PTEN undergoes dephosphorylation of its COOH-terminal tail and is recruited to the cell-cell junction by MAGI-2. Membrane-localized PTEN then mediates the dephosphorylation of its target substrate, PIP3. After releasing from the MAGI-2 complex, PTEN may either be degraded rapidly through a proteosome-dependent pathway or undergo phosphorylation and revert back to the pool of stable PTEN in the cytoplasm.

Obviously, there are still several outstanding questions as to how PTEN phosphorylation is regulated. For example, the physiological signals that are responsible for activating PTEN have yet to be identified. It still remains to be determined whether PTEN can undergo self-dephosphorylation. Also, are there other PTEN kinases in addition to the reported protein kinase CK2 (21)? Finally, it is crucial to understand whether the interaction between PTEN and MAGI-2 is necessary for tumor suppression. In fact, whether MAGI-2 possesses tumor suppressor function has not yet been determined. Developing effective means to disrupt MAGI-2-PTEN interaction will certainly address some of these exciting questions.

[4] Unpublished observations.

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