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13. ABSTRACT ***(Maximum 200 Words)***

The purpose of the research project of this grant is to study the role of phosphorylation on the regulation of PTEN, a tumor suppressor localized on a chromosome region frequently deleted in various cancers including prostate cancer.

Preliminary data presented in the research proposal indicated that PTEN is a phosphoprotein. In this year the major phosphorylation sites of PTEN have been mapped in the C-terminus domain (the tail). It has been demonstrated that the phosphorylation of the PTEN tail regulates its protein stability and its activity. This results made us propose a model in which the PTEN can be found in two different states, in a more inactive but more stable state when is phosphorylated on the tail or in more active but more unstable when is unphosphorylated.

In the next year we hope to identify the signaling that results in phosphorylation of the PTEN tail. The tail kinases by regulating PTEN can also be implicated in cancer. As stated on the grant proposal, 17% of prostate tumors have lost PTEN protein, however PTEN may be deregulated in those tumors which still retain PTEN protein. A certain percentage of tumors may have deregulation of PTEN tail kinases.

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Introduction

The PTEN gene is localized on chromosome 10q23 region, a locus frequently targeted on a variety of cancers including prostate cancer (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997). Furthermore, PTEN heterozygous mice can develop prostate hyperplasia and some percentage can develop prostate tumors at a latter age (Di Cristofano et al., 1999; Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998) . Thus, PTEN is a tumor suppressor probably involved on prostate cancer.

The tumor suppressor function of PTEN has been linked to its ability to act as a lipid phosphatase and in so doing antagonizing the phosphatidylinositol-3-kinase (PI3K) (Cantley and Neel, 1999; Ramaswamy et al., 1999).

The purpose of this research proposal is to find modes of regulation of PTEN. Specifically, the goals of this grant are to identify PTEN phosphorylation sites and test whether these sites play a role on the tumor suppressor function of PTEN.

The significance of this studies to prostate cancer is that by finding modes of regulation of PTEN it could be possible to undercover another deregulated pathway in cancer that could lead to PTEN deregulation giving the same deleterious effects.

BODY

In the research proposal there were three aims proposed:

Aim 1. Identification of PTEN in vivo phosphorylation sites.

This part of the grant has been completed successfully. The phosphorylation sites were mapped by two methods:

1. U20S cells were transfected with a construct encoding HA-PTEN;WT or C-terminus deletion mutant versions of this construct, cells were then metabolically labeled with ^{32}-P and the phosphorylation status was analyzed. Using this method most of the phosphorylation of PTEN were localized on the last 50-aminoacids (the tail). Within the tail, alanine substitution of two serine/threonine clusters were made and its phosphorylation status was analyzed using the same method. The cluster of serine/threonines localized between residues 380 to 385 was found to be phosphorylated. Finally, alanine substitutions of this cluster combined with alanine substitution of serine 370 (see bellow) yielded a protein that lost most f the phosphorylation.
2. 30 plates of p100 U20S cells were transfected with a plasmid encoding HA-PTEN. PTEN was immunoaffinity purified with an anti-HA column. The protein was subsequently runned on a acrylamide gel and after proteolytic digestion tryptic peptides were analyzed in the Harvard Microsequencing facility by liquid chromatography coupled with tandem mass spectroscopy (LC/MSMS). Using this method 69% of the PTEN protein sequence was covered and serine 370 was found to be phosphorylated. The last tryptic peptide containing the 380 serine/threonine cluster was not recovered.

Aim 2. To determine whether there is regulated or constitutive phosphorylation of PTEN phosphoacceptor sites.

In order to pursue this second aim we are in the process of generating phosphospecific antibodies.

These antibodies will be a very powerful tool to analyze whether serine 370 or serine 380 phosphorylation are regulated or constitutive.

In the future, these antibodies, if proven to work on endogenous protein, could also be used to analyze the PTEN phosphorylation status of the prostate tumors that are positive for PTEN protein.

Aim 3. To determine the functional consequences of PTEN phosphorylation.

This part of the research proposal has also been successfully completed.

Recently it has been demonstrated that the PTEN tail while not necessary for PTEN function its necessary to maintain its protein stability. In this study what it was found is that is specifically the phosphorylation of the PTEN tail what is necessary for the stability PTEN. A series of alanine substitutions single or in combination of the PTEN phosphorylation sites were made. The different plasmids were transfected in 7860 cells and the half-life of the different PTEN forms were analyzed. The results demonstrated that phosphorylation of the PTEN tail makes the protein more stable.

Our system for analyzing PTEN function on cell proliferation is the negative PTEN cell line 7860. In these cells PTEN induces a G1 arrest that can be easily detected by FACS analysis after transfection. The plasmids encoding the different forms of PTEN were transfected in 7860 cells and analyzed for its effect on cell cycle progression. Interestingly, alanine substitutions were found to positively regulate PTEN suggesting that the tail phosphorylation plays a negative role of on PTEN function.

Training

The training that I have received during this year can be summarized in the following:

(a) Seminars presented within the Dana-Farber Cancer Institute. During this year of training I have presented my work in the Cancer Biology Seminar Series where the audience involves all the labs within the institute. I have also presented my work on a more specialized joint meetings that include labs that are experts on tumors suppressors including the labs of Dr. David Livingston, Dr. William Kaelin and others.

(b) Conferences attended. (1). Last October, I attended the Cohlrain meeting held annually during three days and that includes experts groups on tumor suppressors from the Boston area. (2) I have attended to the Gordon conference on Second messengers and protein phosphorylation where the presenters where experts on protein phosphorylation and on phosphatases. In this last meeting I presented a poster and I cold get some input on my work from experts in the field.

(c) My technical skills and my intellectual knowledge have been extensively broaden Under Dr. Sellers supervision I have been able to develop new techniques and to trouble-shoot lab protocols. Also we have been able to discuss the project on a daily basis. I have also followed closely the cancer research field and prostate cancer in particular.

Key research accomplishments

- Mapping of PTEN phosphorylation sites
- Determination that PTEN Tail phosphorylation plays an important role on protein stability
- Determination that PTEN Tail phosphorylation negatively regulates PTEN function

Reportable outcomes

- **Manuscripts:**
  Vazquez, F., Ramaswamy, S., Nakamura, N., and Sellers, W. R. (2000). Phosphorylation of the PTEN tail regulates protein stability and function. Mol Cell Biol *20*, 5010-8

  Vazquez, F., and Sellers, W. R. (2000). The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3- kinase signaling. Biochim Biophys Acta *1470*, M21-35

- **Presentation:**
  Francisca Vazquez. Regulation of PTEN by phosphorylation. Colhrain meeting. October 1999.

- **Funding applied for based work supported by this award:**
  R01 CA85912-01. Functional analysis of the PTEN Tumor Suppressor Protein

Conclusions

The conclusion of this research project is that PTEN is phosphorylated on the tail and that this phosphorylation regulates both the protein stability and the activity of PTEN. A model is proposed were PTEN can be found in two different forms; (1) phosphorylated on the tail, more stable but less active and (2) unphosphorylated on the tail, more active but less stable.

These results are potentially very important because there could be a percentage of tumors that have not lost PTEN protein but may have lost its regulation. By finding the pathway that regulates PTEN we may be able to detect, and potentially treat, the "wrongly regulated" PTEN.

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Phosphorylation of the PTEN Tail Regulates Protein Stability and Function

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The *PTEN* gene is a tumor suppressor localized in the frequently altered chromosomal region 10q23. The tumor suppressor function of the *PTEN* protein (PTEN) has been linked to its ability to dephosphorylate the lipid second-messenger phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate and, by doing so, to antagonize the phosphoinositide 3-kinase pathway. The PTEN protein consists of an amino-terminal phosphatase domain, a lipid binding C2 domain, and a 50-amino-acid C-terminal domain (the "tail") of unknown function. A number of studies have shown that the tail is dispensable for both phosphatase activity and blocking cell growth. Here, we show that the PTEN tail is necessary for maintaining protein stability and that it also acts to inhibit PTEN function. Thus, removing the tail results in a loss of stability but does not result in a loss of function because the resultant protein is more active. Furthermore, tail-dependent regulation of stability and activity is linked to the phosphorylation of three residues (S380, T382, and T383) within the tail. Therefore, the tail is likely to mediate the regulation of PTEN function through phosphorylation.

The *PTEN* gene was cloned as a candidate tumor suppressor gene from the chromosome 10q23 region, a locus frequently targeted for genetic loss in tumors (24, 26, 42). Somatic inactivation of both *PTEN* alleles and loss of heterozygosity have been demonstrated in a number of tumors including glioblastoma, melanoma, and prostate, breast, and endometrial carcinomas (reviewed in reference 46). Germ line *PTEN* mutations are associated with the development of the related dominantly inherited disorders known as Cowden disease and Bannayan-Zonana syndrome (28–30, 34). These disorders are characterized by the presence of benign hamartomas of the skin, intestinal tract, and central nervous system and by an increased incidence of cancers of the thyroid and breast (28, 29, 34). Similarly, heterozygous *PTEN* mice develop a variety of tumors and proliferative lesions of multiple tissues (10, 11, 37, 43).

Reconstitution of PTEN expression to certain PTEN null cells results in an increase in the population of cells in the G_1 phase of the cell cycle (13, 23, 39); in other PTEN null cells it results in the induction of apoptosis or anoikis (9, 25, 32). Accumulating evidence suggests that these functions are linked to the lipid phosphatase activity of PTEN, which allows PTEN to antagonize the phosphatidylinositol 3-kinase (PI3K) pathway (reviewed in references 4 and 46). A number of downstream targets of phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate including the serine-threonine kinase Akt, BTK, SGK, and $p70^{S6K}$, have been identified (6, 12, 18–20, 27). Akt, in particular, appears to play a role in both proliferative and apoptotic signals. Constitutive activation of Akt has been found in cells that lack functional PTEN, and PTEN can inhibit Akt kinase activity in cells. A number of downstream targets of Akt have been described and include GSK3, BAD, caspase-9, IKKα, and the forkhead transcription factors FKHR, FKHRL1, and AFX (2, 3, 5, 7, 8, 21, 36, 44). Our group has recently found that forkhead transcription factors are inactive in PTEN null cells and that reconstitution of FKHR activity, in the absence of PTEN, can induce both cell cycle arrest and apoptosis in susceptible PTEN null cells (N. Nakamura, S. Ramaswamy, F. Vazquez, and W. Sellers, submitted for publication).

Each molecular constituent of the PI3K pathway, such as receptor tyrosine kinases, PI3K, and Akt, is subjected to regulation of its activity. Likewise, it has been speculated that PTEN might be regulated, but to date evidence of such regulation has remained elusive (4). In keeping with the idea that PTEN might be regulated, protein phosphatases in general are regulated by a number of mechanisms including phosphorylation, second messengers, regulatory subunits, subcellular localization, dimerization, and binding to inhibitory proteins (reviewed in references 1 and 17).

The PTEN protein contains the signature motif (HCXX GXXR) of the family of protein tyrosine phosphatases and dual-specificity phosphatases. The PTEN crystal structure shows that PTEN consists of an amino-terminal phosphatase domain (PD; residues 7 to 185), which includes the phosphatase signature motif, and a lipid binding C2 domain that extends from residues 186 to 351. C2 domains, named for homology to a domain found in protein kinase C (PKC), have been identified in a number of proteins involved in signal transduction or membrane trafficking such as PKC, $cPLA_2$, phospholipase Cs, and synaptotagmins (reviewed in reference 40). C2 domains can play a role in mediating Ca^{2+}-dependent lipid interactions. However, the C2 domain of PTEN is unlikely to bind Ca^{2+}, and its in vitro binding to lipids is independent of Ca^{2+} (22). The last 50 amino acid residues (354 to 403) (referred to herein as the "tail") were not crystallized, and structural prediction programs fail to identify regions of secondary structure. The function of this domain and its relation-

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ship in three dimensions to the remainder of the protein remain unknown.

The PTEN tail is dispensable for PTEN phosphatase activity and for activity in a number of cellular assays including soft-agar colony suppression assays (14, 22; S. Ramaswamy and W. R. Sellers, unpublished data). Here we show that the tail is necessary for maintaining PTEN stability. However, deletion of the tail also results in an increase in activity as measured by the ability of PTEN to induce a G_1 arrest or to induce the transcriptional activity of FKHR. Thus, deletion of the tail does not result in a loss of PTEN function because, while unstable, the resultant protein is more active. We further demonstrate that the tail is a site for PTEN phosphorylation and that phosphorylation of the tail regulates both PTEN stability and activity.

MATERIALS AND METHODS

Plasmids. pCD19, pSG5L, pSG5L-HA-PTEN, pSG5L-HA-PTEN;1-393, pSG5L-HA-PTEN;1-373, pSG5L-HA-PTEN;1-353, pSG5L-HA-PTEN;1-343, pSG5L-HA-PTEN;1-336; pcDNA3-Flag-FKHR, and pGL3-promoter-FasL were described previously (39, 41, 44, 45; Nakamura et al., submitted).

pSG5L-HA-PTEN;360ΔA, pSG5L-HA-PTEN;S370A, pSG5L-HA-PTEN;A4, pSG5L-HA-PTEN;S380A, pSG5L-HA-PTEN;T382A, pSG5L-HA-PTEN;T383A, pSG5L-HA-PTEN;S385A, pSG5L-HA-PTEN;A3, and pSG5L-HA-PTEN;D3 were generated by site-directed mutagenesis using single-stranded DNA generated from pSG5L-HA-PTEN (Muta-Gene; Bio-Rad). The oligonucleotides used for site-directed mutagenesis are the following: 5′-CACCAGATGTggccGACA ATGAAC-3′ (PTEN;S370A), 5′-GATCATTATAGATATgCTGACgCCgCgGA CgCaGATCCAGAGAATGAAC-3′ (PTEN;A4), 5′-CTGATCATTATCgGaTAT gCaGACACaACTGACTCTG-3′ (PTEN; S380A), 5′-GATCATTATcgaTATT CTGACgcaACTGACTCTG-3′ (PTEN;T382A), 5′-GATATTCTGACACCgcg GACTCTGATC-3′ (PTEN;T383A), 5′-GATATTCTGACACtACaGACgcaGA TCCAGAG-3′ (PTEN:S385A), 5′-GATCATTATAGATATgCTGACgCCgCgG ACTCTGATCCAGAG-3′ (PTEN;A3), 5′-GATCATTATcGATATgaTGACga CgaTGACTCTGATCCAG-3′ (PTEN;D3).

Antibodies and immunoblotting. HA-11 (Babco), antihemagglutinin (HA) antibody was used for immunoblotting at 1:1,000; C54 anti-PTEN serum was previously described and was used at 1:1,000 dilution (39).

Cells were washed in phosphate-buffered saline, and cellular proteins were extracted in TNN buffer (150 mM NaCl, 50 mM Tris [pH 7.4], 0.5% NP-40) for 20 min at 4°C. Lysates were cleared by centrifugation, and proteins were separated by gel electrophoresis. Immunoblots were obtained essentially as described previously (39). Briefly, membranes were blocked in Tris-buffered saline–0.05% Triton X-100 (TBS-T)–4% (wt/vol) milk for 1 h at room temperature (RT). Membranes were then incubated with primary antibodies diluted in TBS-T–4% (wt/vol) milk for 1 h at RT. Subsequently, membranes were washed with TBS-T and incubated with horseradish peroxidase secondary antibody (1:20,000; Pierce Chemicals) diluted in TBS-T–4% (wt/vol) milk. Membranes were washed in TBS-T, and bound antibody was detected by enhanced chemiluminescence (Pierce Supersignal).

Cell lines, cell culture, and transfection. 786-0 and ACHN renal carcinoma cells and U2-OS osteosarcoma cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 4,500 mg of glucose/ml, 2 mM L-glutamine, 10% fetal clone (HyClone), and penicillin and streptomycin and were maintained at 37°C in a humidified 10% CO_2 atmosphere. 786-0 cells were transfected using Fugene reagent (Boehringer Mannheim), and U2-OS cells were transfected using calcium phosphate (BBS method), as previously described (39).

Pulse-chase labeling. 786-0 cells were transfected with various pSG5L-HA-PTEN plasmids and split into p60 plates. Forty hours after transfection, cells were washed twice with methionine-free DMEM and then incubated for 45 min in methionine-free DMEM with 10% dialyzed fetal bovine serum (DFBS) (Gibco BRL). Cells were then incubated for 45 min with methionine-free DMEM–10% DFBS containing [^{35}S]methionine (150 μCi/ml) (NEN Life Science Products). The medium was then replaced with complete medium. HA epitope-tagged proteins were isolated by anti-HA immunoprecipitation and resolved on a 7.5% polyacrylamide gel. The labeled protein present at each time point was quantified by phosphorimaging and normalized to the amount of protein present at the zero-time point.

Cell cycle assays. Cell cycle assays were performed essentially as previously described (39). Briefly, 786-0 cells were cotransfected with 4 μg of pCD19 plasmid along with pSG5L vector or the relevant pSG5L-HA-PTEN wild-type or mutant plasmid. Forty hours after transfection the cells were harvested with trypsin, stained with fluorescein isothiocyanate-conjugated anti-CD19 antibody (CalTag), fixed in 70% ethanol, and stained with propidium iodide in the presence of RNase A. The cell cycle profile of the CD19-positive cells was determined by two-color fluorescence-activated cell sorting (FACS). Data are shown as the percentages of increase in the G_1 population. This was determined by dividing the absolute percentage difference between the vector control and the experimental data point by the percentage of G_1 cells in the vector and then multiplying by 100.

Luciferase reporter assays. FKHR transactivation assays were performed essentially as described previously (Nakamura et al., submitted). Briefly, cells were transfected in 12-well plates with 0.25 μg of the FasL luciferase and β-galactosidase reporter plasmids, 0.5 μg of pCDNA3-FHKR, and various amounts of pSG5L-HA-PTEN. Cells were lysed 40 h after transfection using 1× reporter lysis buffer by following the manufacturer's instructions (Promega). Luciferase and β-galactosidase activities were measured as described previously (41). Luciferase activity was normalized to β-galactosidase activity. Fold activation was calculated by dividing the normalized luciferase activity by the normalized activity obtained in the presence of the vector and reporter plasmid alone.

Metabolic labeling, proteolytic digestions, and phosphoamino acid analysis. ACHN or transfected U2-OS cells were washed twice with phosphate-free DMEM. Then the medium was changed to a mixture of phosphate-free DMEM, 10% DFBS, and 1 mCi (endogenous) or 200 μCi (transfected) of [^{32}P]orthophosphate (NEN Life Science Products)/ml, and the cells were incubated from 2 to 4 h. Labeled proteins were isolated by immunoprecipitation using HA-11 (transfected) or C54 (endogenous) antibodies, resolved by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. The phosphorylated proteins were visualized by autoradiography.

Proteolytic digestions were done essentially as described previously (38). Membrane pieces containing phosphoproteins were excised, washed with double-distilled water (ddH_20), and blocked with 0.5% polyvinylpyrrolidone MW360 (PVP-360) in 100 mM acetic acid for 30 min at 37°C. Digestion was performed with 5 μg of sequencing-grade trypsin (Promega) overnight at 37°C. Peptides were twice lyophilized to dryness and washed with ddH_20. Peptides were then resuspended in a small volume of Laemmli sample buffer and then resolved in 16.5% Tris-Tricine gels.

For phosphoamino acid analysis a fraction of the tryptic digested peptide was hydrolyzed in 5.7 N HCl. The resulting amino acids were then lyophilized, washed with ddH_20, and resuspended in a small volume of ddH_20. Samples were then spotted on thin-layer chromatography (TLC) plates (EM Science) together with 1 μg of cold phosphoamino acid standards (Sigma). Phosphoamino acids were then resolved by electrophoresis at pH 1.9 in a buffer containing 2.5% (vol/vol) formic acid (88%) and 7.8% (vol/vol) glacial acetic acid for 45 min at 800 V in the first dimension and by chromatography in the second dimension in a buffer containing 70% (vol/vol) 2-propanol and 15% (vol/vol) HCl. Cold phosphoamino acid standards were visualized by developing the TLC plates with 0.2% (wt/vol) ninhydrin in acetone and baking them at 65°C until color developed.

RESULTS

The PTEN tail modulates PTEN stability. Recently the crystal structure of PTEN has been solved from residues 7 to 353 (eliminating an internal loop of residues 286 to 309). This truncated protein has in vitro lipid phosphatase activity comparable to that of full-length PTEN (PTEN;WT) and can induce apoptosis in LNCaP cells to the same extent as the wild type (14, 22). Similarly, our group mapped the minimal in vivo functional domain of PTEN by C-terminal and N-terminal deletion mutations (S. Ramaswamy and W. R. Sellers, unpublished data). We found that a truncated PTEN protein of residues 10 to 353 retained protein and lipid phosphatase activity in vitro and was able to induce a G_1 arrest in cells. Furthermore, PTEN;1-353 was comparable to PTEN;WT in suppressing soft-agar colony formation in PTEN null renal carcinoma cells (786-0 cells) (S. Ramaswamy and W. R. Sellers, unpublished data). These results indicate that the last 50 amino acids of PTEN are not necessary for lipid or protein phosphatase activity or for its ability to inhibit proliferation or induce apoptosis. For simplicity we refer to these last 50 residues as the PTEN tail (Fig. 1A).

In our experiments we noted that PTEN;1-353 was produced at markedly reduced steady-state levels compared to PTEN;WT. To determine whether the changes in the steady-state protein levels were related to changes in protein stability, 786-0 cells (PTEN null) transiently transfected with plasmids encoding PTEN;WT or PTEN;1-353 were pulse-labeled with [^{35}S]methionine for 45 min. HA-PTEN and HA-PTEN;1-353 were recovered by immunoprecipitation and detected by autoradiography. In these experiments the half-life of PTEN;1-353

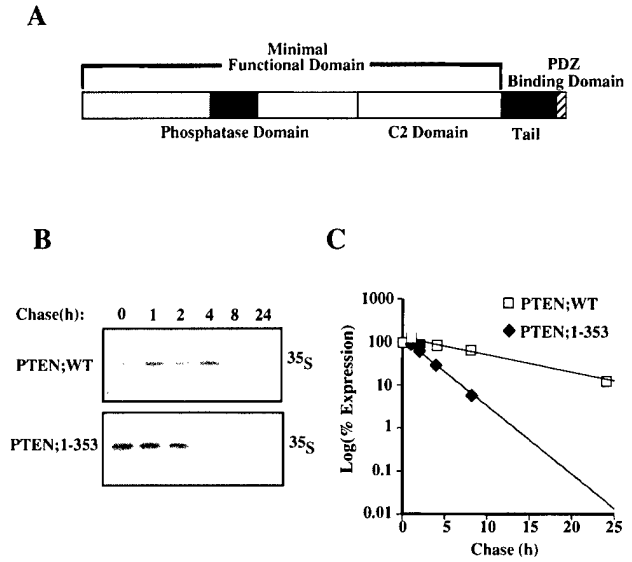

FIG. 1. The PTEN tail is required for protein stability. (A) Schematic representation of the PTEN protein. Dark gray box, phosphatase signature motif (HCXXGXXR); light gray box, PD; white box, C2 domain; black box, C-terminal 50-residues (PTEN tail). The minimal domain that is functional as a growth suppressor is shown. (B) Pulse-chase analysis of PTEN;WT and PTEN;1-353. 786-0 cells were metabolically labeled with [^{35}S]methionine for 45 min. The medium was then replaced with complete growth medium at time zero, and cells were harvested at the indicated times. HA-PTEN and HA-PTEN;1-353 were immunoprecipitated from labeled cell extracts, separated by gel electrophoresis, and detected by autoradiography. (C) Log plot of the percentage of the time-zero protein remaining at the times indicated on the *x* axis. [^{35}S]methionine-labeled, immunoprecipitated protein (B) was quantified by phosphorimager analysis.

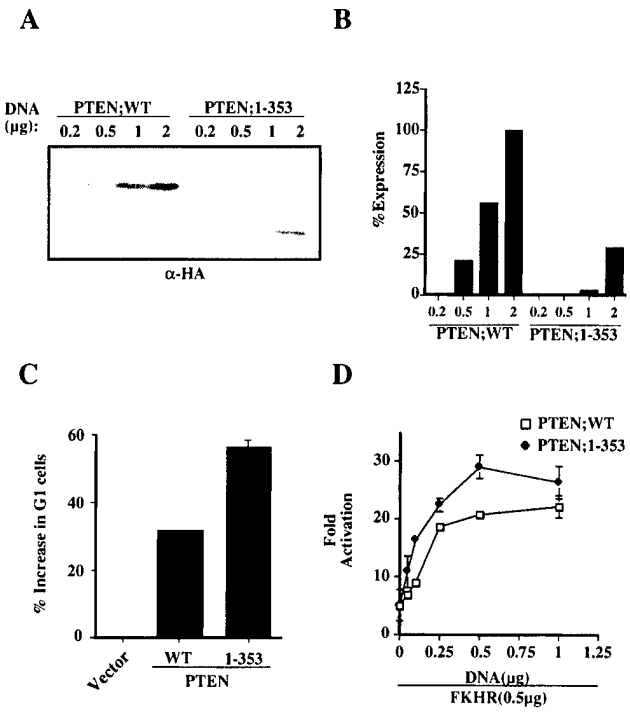

FIG. 2. The PTEN tail is an inhibitory domain. (A) Steady-state protein levels of PTEN;WT and PTEN;1-353. 786-0 cells were transfected with the indicated amounts of plasmids encoding HA-PTEN;WT or HA-PTEN;1-353. Forty hours after transfection, cell lysates were prepared and separated by gel electrophoresis. HA-PTEN and HA-PTEN;1-353 were detected by anti-HA immunoblotting. (B) Quantification of the immunoblot shown in panel A. The radiograph was digitized, and the relative protein quantities were determined using ImageQuant software. The results are expressed as a percentages of PTEN;WT at 2 μg of input plasmid DNA. (C) Induction of G_1 arrest by PTEN;WT and PTEN;1-353. 786-0 cells were cotransfected with a plasmid encoding the cell surface marker CD19 (pCD19) along with plasmids encoding PTEN;WT (0.5 μg) or PTEN;1-353 (2 μg). Forty hours after transfection, the cell cycle distribution of the CD19-positive cells was determined by FACS analysis. Shown are the means and standard errors of duplicate experiments. These data are representative of three independent experiments. (D) Induction of FKHR transcriptional activity by PTEN;WT and PTEN;1-353. 786-0 cells were transfected with a FasL promoter luciferase reporter plasmid and a plasmid encoding FKHR in combination with the indicated amounts of plasmid encoding PTEN; WT or PTEN;1-353. Forty hours after transfection, luciferase activity was determined as described in Materials and Methods. Shown are the means and standard errors of the fold activation relative to the activity obtained with the reporter alone.

was found to be reduced by more than fourfold compared to that of PTEN;WT (Fig. 1B and C). In keeping with these results, it was recently reported that the steady-state level of PTEN;1-351 is reduced compared to that of PTEN;WT when the protein is produced by transfection in COS-7 cells (14). Together these data suggest that the PTEN tail, while not required for the functional activity of the protein, is required for maintaining stability.

The tail domain modulates PTEN biological activity. As a result of the reduced half-life, PTEN;1-353 is produced at significantly lower levels than PTEN;WT. In order to determine whether the resulting decrease in protein production results in reduced activity, PTEN;1-353 and PTEN;WT were compared in a cell cycle arrest assay that reflects the ability of PTEN to act as a lipid phosphatase (39). 786-0 cells were transfected with increasing doses of plasmids encoding PTEN;WT and PTEN;1-353 along with a plasmid encoding the cell surface marker pCD19. The cell cycle distribution of the CD19-positive cells (as a marker of transfection) was determined by staining with fluorescein isothiocyanate-conjugated anti-CD19 and propidium iodide followed by two-color FACS. Surprisingly, at equivalent input plasmid concentrations PTEN;1-353 reproducibly induced a greater increase in G_1 than PTEN;WT (data not shown). Next, the activities of PTEN;WT and PTEN;1-353 were compared when the proteins were produced at similar steady-state levels. Plasmid titration indicated that equivalent protein levels were obtained at 2 μg of PTEN;1-353 and 0.5 μg of PTEN;WT (Fig. 2A and B). At these levels PTEN;1-353 induced a significantly more robust G_1 arrest (Fig. 2C).

Forkhead transcription factors are targets of Akt regulation both in mammalian cells and in *Caenorhabditis elegans* (2, 3, 15, 21, 33, 35). Akt phosphorylation creates 14-3-3 binding sites and results in the cytoplasmic localization of forkhead proteins. Furthermore, recent data in our laboratory have shown that in PTEN null cells FKHRL1 and exogenously expressed FKHR are retained in the cytoplasm and that exogenously expressed FKHR fails to activate transcription. Coexpression of wild-type PTEN, but not a PTEN mutant lacking lipid phosphatase activity, with FKHR restores cytoplasmic localization and FKHR transactivation as measured with a 3XIRS promoter or FasL promoter luciferase reporters (Nakamura et al., submitted). Thus, FKHR transcriptional activity requires PTEN function. The induction of FKHR transcriptional activity by PTEN is also dose dependent, as shown in Fig. 2D.

We next compared PTEN;WT and PTEN;1-353 in a FKHR transcriptional activation assay. Consistent with the results obtained in the cell cycle assay (Fig. 2C), the ability of PTEN;1-353 to induce FKHR transcriptional activity was enhanced compared to that of PTEN;WT. Furthermore, at every DNA

plasmid concentration tested, PTEN;1-353 induced FKHR activation more efficiently than PTEN;WT, although protein levels were reduced by more than fourfold. These results suggest that the PTEN tail not only plays a role in maintaining its protein stability but also in regulating its biological activity. Specifically, these data suggest that the tail acts to restrict or inhibit PTEN function.

PTEN is a phosphoprotein. The PTEN tail is rich in serine and threonine (28% of the residues) and contains consensus phosphorylation sites for GSK3, PKA, CK1, and CK2. In order to determine whether regulation of PTEN stability and activity might be linked to phosphorylation, we determined whether endogenous PTEN is phosphorylated. To this end, ACHN renal carcinoma cells that contained PTEN were metabolically labeled with [^{32}P]orthophosphate. Labeled lysates were incubated with an anti-PTEN antibody (C54) or a preimmune control. Bound proteins were separated by gel electrophoresis and detected by autoradiography. A ^{32}P-labeled protein of the same molecular weight as PTEN was detected in anti-PTEN immunoprecipitates but not in the preimmune control (Fig. 3A). In separate experiments in which the labeled proteins were transferred to nitrocellulose, this ^{32}P-labeled species comigrated with PTEN, as detected by immunoblotting. These data suggest that endogenous PTEN is phosphorylated. Next, PTEN plus U2-OS cells were transfected with either the empty vector (pSG5L) or pSG5L-HA-PTEN and metabolically labeled with orthophosphate. Labeled cells were lysed, and epitope-tagged proteins were immunoprecipitated with anti-HA antibody. In parallel, endogenous PTEN was immunoprecipitated from lysates prepared from untransfected orthophosphate-labeled U2-OS cells. A phosphorylated protein of 58 kDa was detected in the anti-HA immunoprecipitates from cells expressing HA-PTEN but not in the vector-transfected cells (Fig. 3B). Next, phosphorylated endogenous PTEN and exogenously produced HA-PTEN were digested with trypsin. As shown in Fig. 3C, an identical pattern of phosphotryptic peptides was observed when phosphopeptides were separated by Tris-Tricine gel electrophoresis (16.5% acrylamide). Phosphoamino acid analysis of both endogenous and transfected PTEN proteins showed phosphorylation of serine and threonine residues, while tyrosine phosphorylation was not detected (Fig. 3D). These data suggest that endogenous PTEN is a phosphoprotein, that HA-PTEN produced by transfection is a phosphoprotein, and that these proteins are phosphorylated on the same peptides, predominantly on serine and threonine.

PTEN is phosphorylated within the tail domain. To determine the exact sites of phosphorylation, U2-OS cells were transfected with plasmids encoding a series of PTEN C-terminal deletion mutants and labeled with orthophosphate. ^{32}P-labeled HA-tagged proteins were recovered by anti-HA immunoprecipitation and detected by autoradiography. These experiments revealed that deletion of residues 354 to 403 (the tail) abrogated most PTEN phosphorylation (Fig. 4A). In addition, gel electrophoretic separation of peptides generated by cyanogen bromide cleavage of orthophosphate-labeled HA-PTEN revealed a single phosphorylated peptide consistent in size with the predicted CNBr peptide containing the PTEN tail (data not shown). Next, every serine and threonine in the tail was mutated either singly or in clusters to alanine. These PTEN mutants were transfected into U2-OS cells and labeled with orthophosphate. No single-amino-acid substitution abrogated or significantly reduced the total phosphorylation of PTEN (data not shown). However, the substitution of a serine/threonine cluster, S380, T382, T383, and S385 (the A4 mutant), did significantly alter total PTEN phosphorylation. In addition, mutation of these sites led to the loss of the more

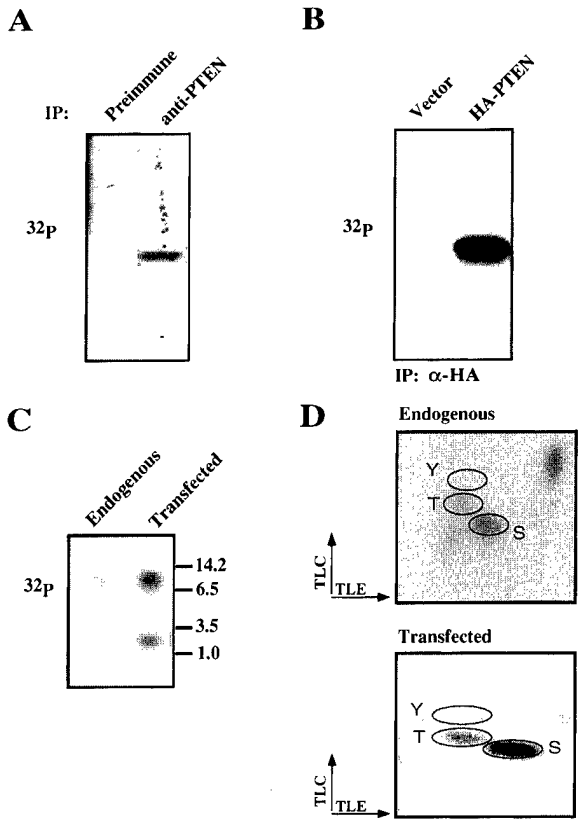

FIG. 3. PTEN is a phosphoprotein. (A) Phosphorylation of endogenous PTEN. Asynchronously growing ACHN cells were metabolically labeled with [^{32}P]orthophosphate for 4 h. Protein extracts were prepared and immunoprecipitated with either preimmune or anti-PTEN (C54) antibody. Bound proteins were resolved by gel electrophoresis, transferred to a nitrocellulose membrane, and detected by autoradiography. Data shown are from the same exposure of the same gel; the lanes were rearranged for clarity. (B) Phosphorylation of exogenous HA-PTEN. U2-OS cells were transfected with the backbone vector or pSG5L-HA-PTEN. Forty hours after transfection cells were labeled with ^{32}P-orthophosphate for 2 h. Protein extracts were prepared and immunoprecipitated (IP) with anti-HA antibody. Bound proteins were resolved and detected as for panel A. (C) Tryptic phosphopeptides of endogenous PTEN and exogenously produced HA-PTEN. The bands corresponding to endogenous PTEN or HA-PTEN (A and B) were excised, digested with trypsin, and resolved on a 16.5% Tris-Tricine gel. Phosphopeptides were detected by autoradiography. (D) Phosphoamino acid analysis of endogenous PTEN and exogenously produced HA-PTEN. Tryptic digest products of in vivo-labeled endogenous PTEN or HA-PTEN were hydrolyzed with acid, and the resulting phosphoamino acids were resolved by two-dimensional thin-layer electrophoresis and detected by autoradiography. Phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards were visualized by ninhydrin staining.

slowly migrating tryptic phosphopeptide (Fig. 4B). This peptide therefore is likely to be peptide 2 (Fig. 4D). In contrast, substitution of alanines for the serine/threonine cluster beginning at S360 (which contains a GSK3 consensus phosphorylation site) had no effect on either the total phosphate incorporated into PTEN or the phosphorylation of the two phosphopeptides detected in Tris-Tricine gels (Fig. 4B). Next, the A4 mutation was combined with a single alanine substitution at a consensus CK2 site (S370) found in peptide 1 of the tail to give the A5 mutation (Fig. 4D). When PTEN;A5 was expressed in U2-OS cells and tested for phosphorylation, ^{32}P labeled protein was not detected despite adequate protein expression (Fig. 4C). Phosphopeptide analysis was not possible because no labeled protein could be excised from the gel. These results indicate that most, if not all, of the PTEN tail

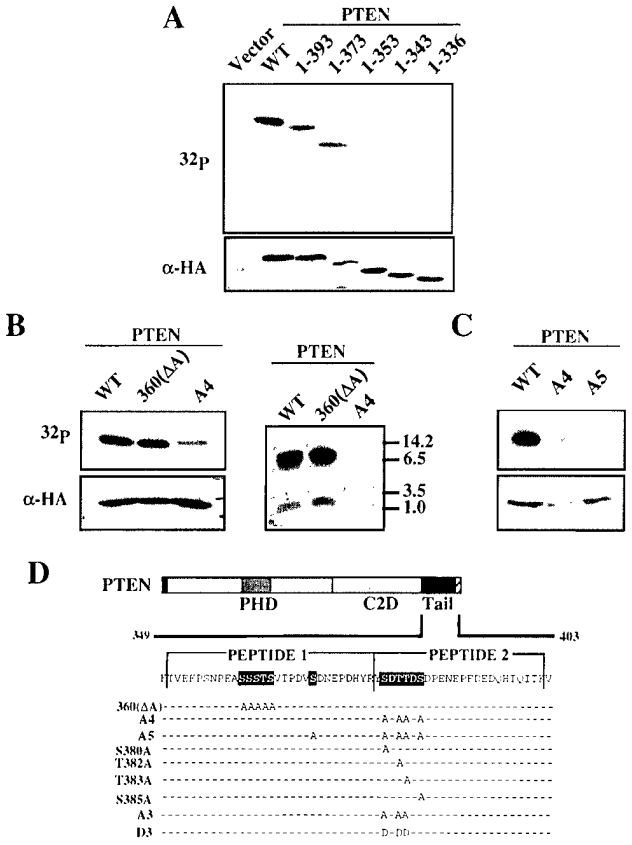

FIG. 4. PTEN is phosphorylated within the tail. (A) Deletion of the tail impairs PTEN phosphorylation. Plasmids encoding either wild-type (WT) HA-PTEN or the indicated C-terminal truncation mutants were transfected into U2-OS cells. Twenty-four hours after transfection, cells were split into T25 flasks and 35-mm plates. Forty hours after transfection, T25 flasks were metabolically labeled with [^{32}P]orthophosphate; anti-HA immunoprecipitates of protein extracts were prepared, and bound labeled proteins were detected by autoradiography (top). In parallel, whole-cell extracts prepared from the 35-mm plates were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-HA antibody (bottom). (B [left] and C) Alanine mutations in the PTEN tail impair phosphorylation. U2-OS cells were transfected with plasmids encoding PTEN;WT or the indicated alanine substitution mutants. Transfected cells were split, replated, grown overnight, and used in parallel for metabolic labeling and for the preparation of whole-cell extracts. [^{32}P]orthophosphate labeling, immunoprecipitation, and autoradiography (top) were performed as for panel A. Anti-HA immunoblotting (bottom) was performed as for panel A. (B [right]) Tryptic phosphopeptides of PTEN tail substitution mutants. Phosphopeptides resulting from the tryptic digestion of either HA-PTEN;WT or the indicated mutant proteins were resolved on Tris-Tricine gels and detected as for Fig. 3C. (D) Schematic representation of PTEN tail substitution mutants used. Dashes indicate amino acids that were not altered. The predicted tryptic peptides of the PTEN tail are shown as peptide 1 and peptide 2. PHD, phosphatase domain; C2D, C2 domain.

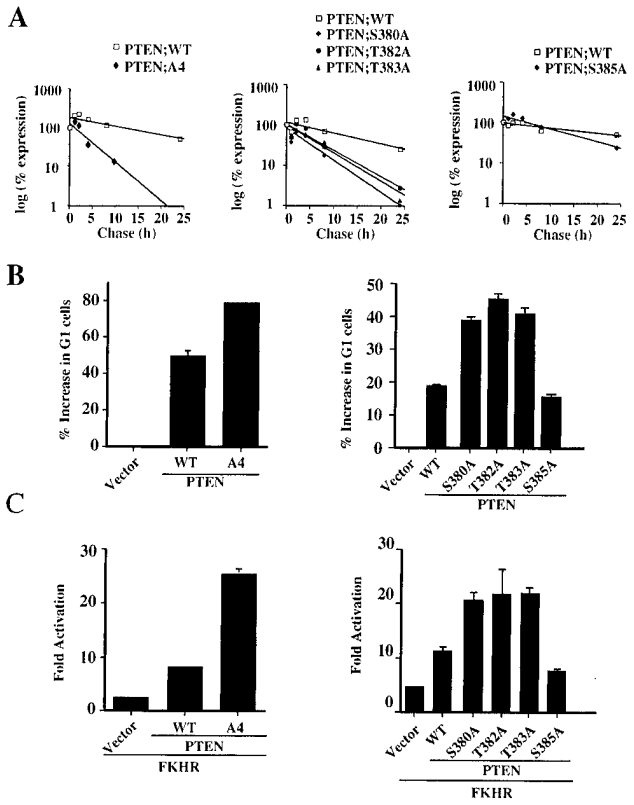

FIG. 5. Mutation of phosphoacceptor sites in the PTEN tail alters protein stability and activity. (A) Protein half-life of PTEN;A4 (left) and single-substitution mutants (middle and right). 786-0 cells were transfected with plasmids encoding HA-PTEN;WT and HA-PTEN;A4 or the indicated single-substitution mutants, metabolically labeled with [^{35}S]methionine, and chased for the indicated times. Data are shown as in Fig. 1C. (B) Increased activity of PTEN;A4 (left) and mutants with at single substitution of S380, T382, or T383 (right) in the cell cycle arrest assay. 786-0 cells were cotransfected with a plasmid encoding CD19 (pCD19) along with plasmids encoding PTEN;WT, PTEN;A4, or single-substitution mutants at concentrations resulting in equivalent protein production (0.5 μg of pSG5L-HA-PTEN;WT and pSG5L-HA-PTEN;S385A and 2.0 μg of pSG5L-HA-PTEN; A4, pSG5L-HA-PTEN;S380A, pSG5L-HA-PTEN;T382A, and pSG5L-HA-PTEN;T383A). Forty hours after transfection the cell cycle distribution of the CD19-positive cells was analyzed as for Fig. 2C. These data are representative of three independent experiments. (C) FKHR transcriptional activity induced by PTEN;A4 (left) and single-substitution mutants (right). 786-0 cells were transfected with a plasmid encoding FKHR along with either the backbone vector or plasmids encoding PTEN;WT, PTEN;A4, or single-substitution mutants as indicated. Forty hours after transfection luciferase activity was measured and the fold activation of FKHR relative to the activity obtained with the reporter alone was calculated as for Fig. 2D. Shown are the means and standard errors of experimental duplicates. These data are representative of two independent experiments.

phosphorylation occurs on serine 370 and one or more sites of the A4 cluster (S380, T382, T383, and S385).

To directly identify the PTEN phosphorylation sites, 2 μg of HA-PTEN isolated by anti-HA immunoaffinity purification was digested with trypsin and analyzed by LC/MSMS. Here, peptide 1 with a phosphoserine at residue 370 was identified. However, peptide 2 was not detectable in either a phosphorylated or unphosphorylated state. No other phosphopeptides were identified (data not shown).

Mutation of the phosphorylation sites in the tail alter PTEN stability and biological activity. To determine whether phosphorylation of one or more of the amino acid residues delineated above has a role in modulating PTEN stability, the relevant phosphorylation site mutants were produced in U2-OS cells by transient transfection and the steady-state levels of HA-PTEN and the phosphorylation mutants were determined by immunoblot analysis. While mutation of serine 370 did not change the steady-state level of PTEN (data not shown), mutation of the S/T cluster (PTEN;A4) resulted in a marked decrease in the steady-state protein level (data not shown). Furthermore, pulse-chase labeling experiments revealed a marked reduction in the PTEN;A4 half-life (Fig. 5A, left).

As deletion of the tail led to an increase in PTEN activity, we next asked if the PTEN;A4 mutant was similarly more active in biological assays. In keeping with the data for the PTEN tail, the PTEN;A4 mutant, while expressed at lower levels, was more active in both inducing a G_1 arrest in PTEN null 786-0

cells (Fig. 5B, left) and inducing FKHR transcriptional activation (Fig. 5C, left). These data suggest that phosphorylation within the A4 cluster is required to maintain stability and is linked to an inhibitory activity of the PTEN tail.

Next, the individual point mutations within the A4 cluster were tested in the same assays of protein half-life and activity. While replacement of serine 385 with alanine did not alter the steady-state PTEN protein levels, mutation of S380, T382, and T383 each reduced both the steady-state protein levels and the protein half-life (Fig. 5A, right, and data not shown). More specifically, the half-life of the PTEN;A4 mutant was reduced more than six-fold compared to that of PTEN;WT. Similarly, mutating serine 380 reduced the half-life by more than fivefold. Mutation of threonines 382 and 383 reduced PTEN half-life by 2.7- to 3-fold. Furthermore, the individual phosphorylation mutants with substitutions S380A, T382A, and T383A, but not S385A, were again more active in inducing a G_1 arrest and in inducing FKHR transcriptional activation (Fig. 5B and C, right). Taken together, these data show that the increased activity associated with deletion of the tail is entirely mimicked by mutations within the A4 cluster, specifically S380, T382, or T383.

The above data raised the possibility that phosphorylation of these three specific residues (S380, T382, and T383) might be required to maintain PTEN in a stable yet relatively inactive state. While mutation of each of these individual residues did not alter total incorporation of ^{32}P into the PTEN protein (data not shown) when the S380, T382, and T383 residues were mutated to alanine (PTEN;A3) incorporation of ^{32}P into PTEN during orthophosphate labeling was reduced (Fig. 6A) and the most slowly migrating tryptic phosphopeptide (peptide 2) was not detectable (Fig. 6B). In keeping with the data for PTEN;A4 and for the individual phosphorylation site mutants (with mutations S380A, T382A, and T383A), PTEN;A3 was found to have a reduced protein half-life, to be expressed at lower steady-state levels, and to be more active than wild-type PTEN in biological assays (Fig. 6C to F).

Aspartic acid substitutions at the phosphorylation sites in the PTEN tail lead to a recovery of PTEN stability. A reasonable interpretation of these results is that the serine/threonine-to-alanine substitutions of PTEN block phosphorylation and thereby alter the stability and activity of PTEN in cells. On the other hand, it is formally possible that mutation of these residues might result in these changes independent of the changes in PTEN tail phosphorylation. To distinguish these possibilities, conversion of the serines/threonines to aspartic acid was used to try and mimic phosphorylation of these residues. As we had noted that mutation of any one of the putative phosphorylation sites altered both stability and activity, it appeared that phosphorylation of all three residues might be required for maintaining PTEN stability. Therefore, we generated a PTEN;D3 cDNA in which codons 380, 382, and 383 encoded aspartic acid.

In contrast to the results obtained with PTEN;A3, PTEN;D3 recovered the expression levels of PTEN;WT, suggesting that a negative charge was enough to maintain protein stability (Fig. 6C). To test this possibility, we performed a pulse-chase experiment to determine if aspartic acid substitution could restore PTEN stability. As shown in Fig. 6D, PTEN;D3 recovered the stability of PTEN;A3 and was similar to PTEN;WT. As expected, when analyzed after orthophosphate labeling, PTEN;D3 like PTEN;A3 was found not to contain phosphopeptide 1 (data not shown). These data argue that the D3 mutation does not restore stability simply be restoring phosphorylation of PTEN at other sites but rather that phosphorylation of the S380 cluster is required for appropriate stability.

Next, we asked whether aspartic acid substitution led to a change in the activity of PTEN in the cell cycle assay and the FKHR transactivation assay. As would be predicted if loss of phosphorylation was responsible for the changes seen in PTEN activity, PTEN;D3 recovered the activity of PTEN;WT and its activity was reduced compared to that of PTEN;A3 (Fig. 6E-F).

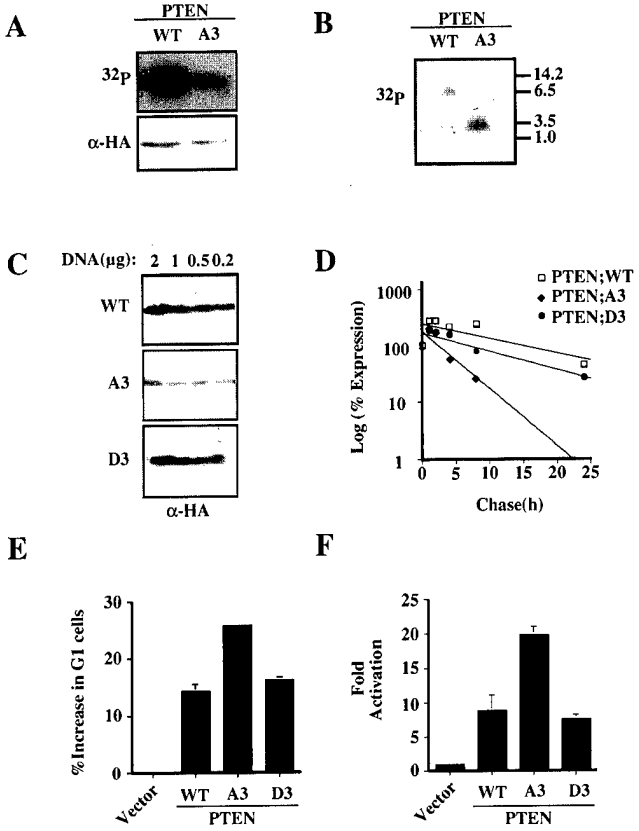

FIG. 6. Aspartic acid substitutions of serine 380, threonine 382, and threonine 383 restore PTEN expression levels and half-life. (A) Substitution mutations of serine 380, threonine 382, and threonine 383 impair PTEN phosphorylation. Plasmids encoding PTEN;WT and PTEN;A3 were transfected into U2-OS cells, and phosphorylation of the resulting HA-PTEN proteins was determined as for Fig. 4A. (B) Phosphorylated tryptic peptides of the PTEN tail substitution mutants. Phosphopeptides resulting from the tryptic digestion of HA-PTEN;WT or HA-PTEN;A3 were resolved on Tris-Tricine gels and detected by autoradiography. (C) Aspartic acid substitution restores HA-PTEN steady-state protein levels. 786-0 cells were transfected with the indicated amounts of plasmids encoding HA-PTEN;WT and HA-PTEN;A3 and HA-PTEN;D3 mutants. Forty hours after transfection whole-cell extracts were analyzed by immunoblotting with anti-HA antibody. (D) Aspartic acid substitution restores PTEN stability. 786-0 cells were transfected with plasmids encoding HA-PTEN;WT, HA-PTEN;A3, or HA-PTEN;D3, metabolically labeled with [^{35}S]methionine, and chased for the indicated time. Data are shown as in Fig. 1C. (E) Cell cycle arrest induced by PTEN;WT or the indicated substitution mutants. 786-0 cells were transfected with plasmids encoding PTEN;WT or the indicated PTEN mutants. Forty hours after transfection the cell cycle distribution of the CD19-positive 786-0 cells was determined as for Fig. 2C. Data are the means and standard errors of experimental duplicates. The data are representative of three independent experiments. (F) FKHR transcriptional activation. 786-0 cells were transfected with the FasL promoter luciferase reporter plasmid and a plasmid encoding FKHR alone or in combination with a plasmid encoding PTEN;WT or the indicated PTEN substitution mutants. Forty hours after transfection the fold activation of reporter activity was determined as for Fig. 2D. The data are the means and standard errors of experimental duplicates. These data are representative of two independent experiments.

DISCUSSION

Recently it was shown that PTEN;1-351 has in vitro lipid phosphatase activity, can inhibit Akt activation, and can sup-

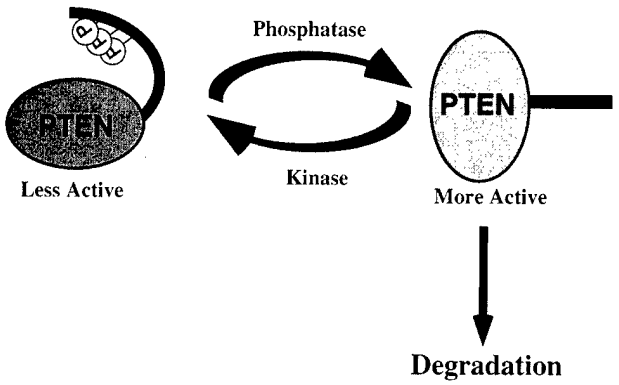

FIG. 7. Model for PTEN regulation by phosphorylation of the tail. The black extension to PTEN represents the tail, and the circles labeled P represent phosphorylated residues within the tail. PTEN phosphorylation on the tail would restrict PTEN activity. Dephosphorylation of the tail would result in an increase in PTEN activity and in its rapid degradation.

press anchorage-independent growth (14). In accordance with these results, we found that PTEN;1-353 inhibits the growth of PTEN null 786-0 cells in soft agar and induces apoptosis in LNCaP cells (S. Ramaswamy and W. R. Sellers, unpublished data). Furthermore, the PTEN crystal structure shows that these residues include the entire PD and a C2 lipid binding domain (22). Together these data suggest that the tail (residues 354 to 403) is not required for biological activity of the protein.

Here, we have shown that deletion of the PTEN tail results in a loss of protein stability. One might expect that loss of stability would lead to a loss of PTEN function; however, as stated above, no requirement for the tail was found in multiple assays of PTEN function. This paradox can be explained by the concomitant loss of an inhibitory activity that maps to the tail region. Thus, the tail contains sequences that are required for protein stability and for negative regulation of PTEN function. This linkage between stability and activity argues for a model in which PTEN is normally found in a stable yet relatively inactive state. In such a model, activation of PTEN would be accompanied by a decrease in protein half-life, presumably reflecting degradation of the active molecule (Fig. 7). Such a mechanism would prevent the untimely or promiscuous activation of PTEN. The linkage between protein activation and protein instability is a common theme in molecular biology. Examples of proteins where the activated form of the protein is unstable include Src, EGFR, PDGFR, and the nuclear receptors RAR and RXR (16, 31, 47).

The tail is also required for PTEN phosphorylation. In vitro mutagenesis revealed specific phosphorylation of S380, T382, and T383. Mutation of these sites led to a loss of stability and a gain in PTEN function. Conversely, aspartic acid mutations of these same residues preserved the protein half-life and the function of PTEN. Together, these data argue strongly that phosphorylation of these residues is required for stability and that the changes in stability are not simply the result of a misfolding secondary to changes in the amino acid residues. Recently others have shown that mutations in the C2 domain can reduce expression levels and protein half-life (14). The mutants are, however, found to be functionally inactive or significantly impaired in biological assays; thus the instability of these mutants might indeed arise as a consequence of protein misfolding (14, 22). On the other hand, the PTEN;A3 mutant, while unstable, is more active, arguing strongly against protein misfolding as a mechanism for instability in this instance.

What is the mechanism by which PTEN phosphorylation regulates protein stability? As pointed out by Georgescu et al. (14) the tail contains two putative PEST sequences (residues 350 to 375 and 379 to 386) implicated in targeting proteins for proteolytic degradation. We have found that the second PEST region is the site of three phosphorylation sites (S380, T382, T383), raising the possibility that this sequence is normally masked by phosphorylation. Arguing against this model, however, is the fact that deletion of the entire tail also results in a shorter half-life. Secondary structure prediction and the results of proteolytic digestion experiments suggest that the tail is a relatively unstructured and presumably flexible region. Thus, one model is that the tail can mask a degradation signal present elsewhere in the PTEN protein when phosphorylated; this signal would be unmasked by dephosphorylation leading to a shift in the position of the tail (Fig. 7).

As an alternative model, the tail might regulate PTEN localization through interactions with the adjacent C2 domain. If so, then stability might simply reflect the localization of PTEN to a subcellular compartment where PTEN degradation can take place. To date we have not seen an obvious effect on the membrane localization of the relevant C-terminal truncations or phosphorylation site mutants (data not shown). Surprisingly, PTEN;1-353 and PTEN;A4 manifestly increased localization to the nucleus (data not shown). Whether this apparent change in localization results from a more rapid degradation of the cytoplasmic component of these mutants or from a true shift in localization is not yet clear. Nonetheless, regulation of localization by tail phosphorylation might play an important role in the regulation of PTEN.

What is the mechanism through which PTEN function is regulated by the tail? There are a number of mechanisms that could account for the inhibitory activity of the tail on PTEN. First, the phosphatase activity itself could be regulated through an allosteric or steric mechanism. To date, however, we have seen no effects of phosphorylation site mutations on intrinsic phosphatase activity (data not shown). Second, the ability of PTEN to gain access to a substrate could be altered either through changes in localization (see above), through changes in the position of the tail, or perhaps through inhibitory interactions with tail-associated proteins. With respect to the last idea, the PTEN tail contains a PDZ binding domain. Given that the PDZ binding sequence (along with the entire tail) is dispensable for the biological function of PTEN, it would seem likely that this domain is linked to the role for the tail that we have put forth. Specifically, the interaction of a PTEN with a PDZ domain-containing protein might be regulated through PTEN phosphorylation.

Are the PTEN phosphorylation events that we have identified constitutive or regulated? Our data are most consistent with the idea that PTEN exists in a predominantly phosphorylated state and that dephosphorylation of the S380 cluster is a regulated event. While PTEN runs as a single band under standard sodium dodecyl sulfate-gel electrophoresis conditions, on two-dimensional gels multiple isoforms of PTEN can be distinguished based on differences in the isoelectric point (data not shown). While it is likely that these forms represent different PTEN phosphoisoforms, how these forms are related to each phosphorylation site or to PTEN activity or stability is not yet known. Our model suggests that PTEN function is regulated by the balance between a kinase and a phosphatase. It is possible that the kinase constitutively phosphorylates PTEN and that a phosphatase regulates the activity. An intriguing possibility is that PTEN activates itself through autodephosphorylation maintaining a constant loop of activity. Identification of the kinase and phosphatase activities respon-

sible for the regulation of these sites should provide further insights into how regulation of PTEN is achieved.

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Minireview

The PTEN tumor suppressor protein: an antagonist of phosphoinositide 3-kinase signaling

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1. Introduction

PTEN/MMAC-1/TEP-1 (referred to hereafter as *PTEN*) is a tumor suppressor gene that maps to the 10q23 interval, a chromosomal region frequented by loss-of-heterozygosity (LOH) and, thus, of long-standing interest to those studying somatic mutations in human tumors. In addition, linkage analysis has previously implicated this locus in the inheritance of Cowden disease, a rare hereditary breast and thyroid cancer predisposition syndrome. *PTEN* was cloned by three groups, and analysis of somatic mutations in tumors and of the germline *PTEN* allele in Cowden disease strongly suggests that *PTEN* is a tumor suppressor in these diseases (as will be discussed below) [1–3].

The protein product, PTEN, has homology to dual-specificity phosphatases. Recent evidence suggests that PTEN can function not only as a protein phosphatase, but also as a lipid phosphatase [4]. In particular, PTEN dephosphorylates by-products of phosphoinositide 3-kinase (PI3K) and in so doing functionally antagonizes signaling pathways that rely on PI3K activity.

This review outlines the PI3K pathway and the connections to cell survival and proliferation and the position of PTEN in this pathway. PTEN's role as a protein phosphatase and the significance of somatic and germline PTEN mutations will also be discussed.

2. The phosphoinositide 3-kinase pathway

In multi-cellular organisms, cell viability and growth depend in part upon the state of the microenvironment. Environmental stimuli elicit responses in target cells through specific signaling pathways often by activating transmembrane receptors. Subsequent signal transmission is carried by intracellular messengers as diverse as nitric oxide, STAT proteins, Ca^{2+}, phospholipids, and cAMP.

A number of phosphorylated lipid second messengers derived from the phosphorylation of phosphatidylinositol (PI) have been identified including phosphatidylinositol-4,5-bisphosphate (PI4,5P2), phospatidylinositol-3-phosphate (PI3P), phospatidylinositol-3,4-bisphosphate (PI3,4P2), and phospatidylinositol-3,4,5-trisphosphate (PI3,4,5P3). PI3P is found constitutively in cells and functions in vesicle transport. PI4,5P2 is an abundant lipid which when hydrolyzed gives rise to inositol-1,4,5-trisphosphate a mediator of calcium release. On the other hand, PI3,4,5P3 and PI3,4P2 are evanescent phospholipids that are virtually absent in quiescent cells and are up-regulated after growth factor stimulation (for review see [5]). The production of these phospholipids requires the action of a kinase capable of delivering a phosphate to the D3 position of the inositol head group. The enzymatic phosphoinositide 3-kinase activity required for this phosphorylation was first found in association with a complex of the transforming, src and polyoma Middle T antigen, and, subsequently, in association with the PDGF and CSF-1 receptors [6–8].

2.1. Activation of the PI3K pathway (building the complex)

PI3K is a heterodimeric enzyme consisting of a catalytic subunit (p110) and an associated regulatory subunit, p85. The cloning of a number of catalytic subunits of PI3K has led to the organization of this multi-gene family into three groups (types I, II and III) based upon substrate specificity (for review see [9]). For the purposes of this review, we will focus on the type I class (p110α, p110β, p110δ, p110γ), typified by the originally cloned p110α catalytic subunit.

Activation of PI3K activity can be accomplished through receptor tyrosine kinase activation, through the activation of non-receptor tyrosine kinases or through ras activation. Growth and survival factors bring the PI3K pathway to life by activating receptor tyrosine kinases (see Fig. 1, #1). Receptor activation often accompanied by receptor dimerization, triggers receptor autophosphorylation on tyrosine residues creating docking sites for the SH2 domains of p85. Conformational changes in p85, set in motion by binding of the SH2 domains, and the recruitment of p85/p110 to receptor tyrosine kinases leads to the localized activation of PI3K at the inner surface of the plasma membrane (Fig. 1, #2). (for review see [10]). In the case of the insulin or IGF-1 receptors, this activation requires the phosphorylation and binding of an intermediary, IRS-1, that then binds to p85. Non-receptor tyrosine kinases, such as src

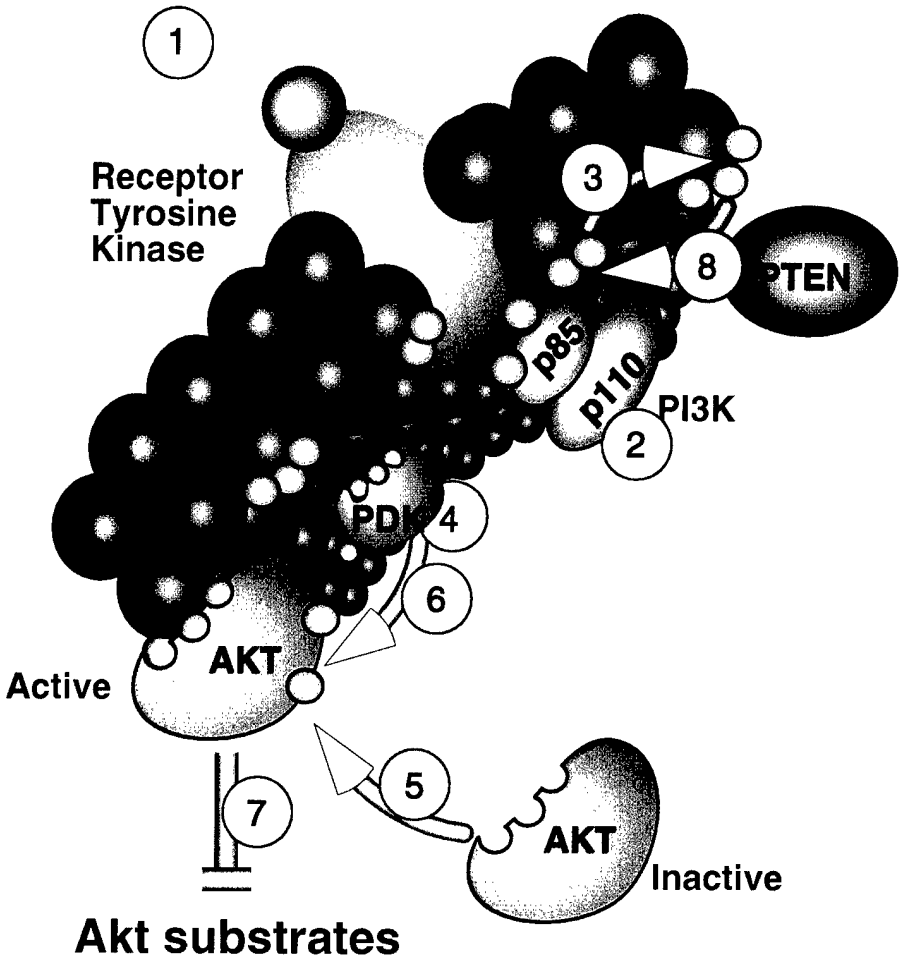

Fig. 1. Activation of the PI3K/Akt pathway. This figure depicts an idealized section of the plasma membrane. Embedded in the lipid bilayer (black balls representing the lipid head groups) is an idealized transmembrane receptor tyrosine kinase (green). Such receptors would often exits as dimers. Phosphorylation events on both proteins and lipid are represented by small yellow balls. 1, Growth or survival factors bind to a target receptor and induce auto tyrosine phosphorylation; 2, PI3K, composed of p85 and p110 subunits is recruited to the receptor through an interaction between p85 and the receptor; 3, active PI3K phosphorylated PI4,5P2 (black ball with two phosphates) on the D3 position generating PI3,4,5P3 (black ball with three phosphates); 4, PI3,4,5P3 serves as a binding site for PDK serine-threonine kinases; 5, PI3,4,5P3 serves as a binding site for Akt serine-threonine kinases; 6, PDK phosphorylates and activates Akt. In this case, phosphorylation and activation appears to require two enzymatic activities PDK1 and PDK2. PDK1 is a distinct gene product. The exact identity of PDK2 is uncertain; 7, active Akt phosphorylates substrates. Typically, such phosphorylations render the substrates inactive; 8, PTEN functions as an antagonist by dephosphorylating the lipid products generated by PI3K activity.

and lck, may recruit p85 via an SH3-mediated interaction. Again, the net effect is to direct the p85/p100 enzyme to the membrane. Finally, PI3K activation is mediated through Ras family members. Here, the generation of activated GTP-bound Ras leads to direct recruitment and activation of the catalytic subunit of PI3K (p110) in the absence of effects on p85 (for review see [11]).

Once brought to the membrane and activated, PI3K specifically phosphorylates the D3 position of the inositol ring of phosphatidylinositides to generate the phosphatidylinositol-3,4-bisphosphate (PI3,4P2) and phosphatidylinositol-3,4,5-trisphosphate (PI3,4,5P3) (Fig. 1, #3). PI3,4P2 and PI3,4,5P3 can be thought of as membrane localized second messengers that serve to recruit certain cytosolic proteins to

the sites of PI3,4P2 and PI3,4,5P3 at the plasma membrane. This recruitment is mediated by a protein–lipid interaction domain known as a Pleckstrin homology domain (PH domain).

2.2. Downstream targets of PI3K activation: a focus on Akt

Among the downstream targets of PI3K activation is the Akt family of serine-threonine kinases. This family consists of Akt-1,-2 and -3. (For simplicity, the group will be referred to as Akt). Akt-1 was initially identified as the cellular homologue of the retroviral oncogene, v-Akt, from the retrovirus AKT8 [12]. Akt proteins contain at the amino-terminus a so-called 'Pleckstrin homology domain' (PH domain). PH domains are a conserved protein–lipid interaction domain that can be found in a wide variety of proteins (for review see [13]). The PH domain of Akt can bind with high affinity to PI(3,4,5)P2, resulting in the translocation of Akt from the cytosol to the plasma membrane and a conformational change in Akt (for review see [14]) (Fig. 1, #5). This conformational change results in the exposure of an activation loop that is then phosphorylated on Thr-308 and Ser-473 by PDK1, a PI3K-dependent serine/threonine kinase and by a distinct unidentified kinase (PDK2), respectively (Fig. 1, #6). The elusive PDK2 activity might, in fact, be PDK1 itself or, alternatively, integrin-linked kinase (ILK) [15,16]. While phosphorylation is necessary for activation, the exact mechanism of activation is not known. The viral oncogene, *v-akt*, subverts the mechanism of Akt activation and renders it constitutive. Here, a fusion of a viral *gag* protein to Akt (creating a gag-akt protein), eliminates the PH domain and directs Akt to the membrane constitutively where it is activated.

Numerous targets of PI3K activation have been described including PDK1 itself, Phospholipase Cζ and the Bruton's tyrosine kinase (BTK) to name but a few (for review see [10]). How specificity and/or selectivity is generated downstream of PI3K is not yet clear. However, it is clear that PH domains and other lipid binding domains exhibit some selectivity with respect to the preference of phospholipids they can bind. This may, in part, begin to explain some aspects of specificity.

2.3. Downstream targets of Akt

When activated, Akt phosphorylates proteins on serine and threonine residues (Fig. 1, #7). The majority of these phosphorylations render the target substrates inactive. Substrates of Akt include GSK3, $p70^{S6K}$, BAD, Caspase 9, 4E-BP1/PHAS-1, IKKα and members of the Forkhead transcription family (FKHRL1, FKHR, AFX) [17–31]. Akt substrates, such as BAD, Caspase 9, FKHR, and FKHRL1 are death-promoting components of apoptotic regulatory pathways and Akt, presumably through inhibition of these targets, can potentiate cell survival in a number of systems (for review see [14]). Akt substrates, such as GSK3 and perhaps FKHR, are linked to the regulation of insulin response. Finally, substrates such as $p70^{S6K}$ kinase and 4E-BP1/PhasI are involved in regulating protein translation in response to mitogens and, indeed, $p70^{S6K}$ activity appears to be required for S phase entry [32]. Regulation of these later substrates, however, is not clearly directly downstream of Akt (see below).

Recently, a new PI3,4,5P3-dependent enzyme was isolated and cloned, PDK1. PDK1 is activated by PI3K and is capable of and required for phosphorylation and activation of Akt (see above). In addition, PDK1 can also phosphorylate and activate $p70^{S6K}$ and isoforms of PKC [33]. Whether there is a further division or a strict division of substrates between Akt and PDK1 remains to be seen.

3. PTEN: an antagonist of PI3K signaling

3.1. PTEN is a lipid phosphatase

The PTEN gene encodes a protein of 403 residues that shares homology to dual-specificity phosphatases [1,2]. PTEN encodes the active site consensus motif HCXXGXXR(S/T) found in all PTPases, and enzymatic activity of recombinant PTEN has been demonstrated against both tyrosine and serine/threonine phosphorylated substrates and against the lipid second messenger, PI(3,4,5)P3 [4,34]. Recombinant PTEN can specifically catalyze the dephosphorylation of the D3 position of inositol and phosphatidylinositol substrates [4]. Furthermore, overexpression

of PTEN in cells reduces the levels of insulin-induced PI(3,4,5)P3 in a dose-dependent manner and PTEN−/− mouse embryo fibroblasts have elevated levels of PI(3,4,5)P3 when compared to their heterozygous counterparts [4,35,36]. Importantly, both tumor-derived and Cowden's disease- derived PTEN mutants have been described that retain protein phosphatase activity, but are devoid of activity against the phosphoinositides [37–39]. These data suggest that the protein phosphatase activity is not sufficient for PTEN tumor suppressor function. This and other evidence, mentioned later, suggest that PTEN acts as a tumor suppressor, at least in part, through its ability to dephosphorylate PI(3,4,5)P3.

3.2. *PTEN inhibits cell cycle progression through PI3K/Akt*

Growth suppression resulting from PTEN reconstitution to PTEN null cells appears to result from distinct outcomes, producing a G1 cell cycle arrest in some cell types, but inducing apoptosis in others. Work from our laboratory and others has demonstrated a role for PTEN in regulating cell-cycle progression, a function that is disrupted by somatic and germline derived mutations of PTEN. Reconstitution of PTEN to PTEN−/− UMG-87 and U178 glioblastoma cell lines and 786-O renal carcinoma cells leads to an accumulation of cells in G1 [38–40]. PTEN mutations that alter the lipid, but not protein phosphatase activity inactivate the cell-cycle arrest function of PTEN or alter growth suppression by PTEN [37–39]. Furthermore, in the setting of a PTEN-induced cell cycle block, while co-expression of wild-type Akt-1 does not override the arrest, a myristoylated form of Akt-1 which is targeted and activate independently of PI3,4,5P3, can override the G1 arrest [38]. These data suggest that PTEN-mediated cell cycle inhibition depends upon inhibition of PI3K and Akt. In keeping with this notion, PDGF-induced mitogenesis requires PI3K activation, and, in the absence of serum, both PI3K and, to a lesser extent, Akt-1, can induce S phase entry in fibroblasts [41,42].

Where does the PI3K/PTEN/Akt pathway intersect with the intrinsic cell cycle machinery? Previous data have linked Akt to the regulation of cyclin D1 and to the regulation of E2F activity [43–45]. On the other hand, two independent studies have found an increase in the cyclin dependent kinase inhibitor p27 and a concomitant decrease in Cdk2 kinase activity after restoring PTEN expression to PTEN null glioblastoma cells [40,46]. Levels of p27 are also reduced in PTEN−/− embryonic stem (ES) cells compared to their wild-type counterparts [47]. These results suggest that upregulation of p27 levels by PTEN might lead to decreased activity of the G1 cell cycle kinases that are needed for S phase entry. Whether altered p27 levels reflect a direct connection between PTEN, Akt and p27 or arise as a result of alterations in cell-cycle kinetics is not known.

Together, the aforementioned data support the idea that ectopic expression of PTEN is sufficient for the induction of a cell-cycle block in certain cells. PTEN heterozygous mice have elevated numbers of S phase and mitotic cells in the prostate and thyroid. PTEN−/− embryos have abnormally high rates of BrDU incorporation, and PTEN−/− ES cells have decreased levels of p27 with abnormal cell-cycle kinetics. Together, these data all derived from the studies of PTEN loss-of-function, suggest a necessary role for PTEN in cell-cycle regulation [35,47,48]. Thus, it is clear that at least one important function of the PI3K/PTEN/Akt pathway is the regulation of cell-cycle entry.

3.3. *PTEN induces apoptosis through the PI3K/Akt pathway*

A number of lines of investigation have, over the past several years, pointed to a critical role for Akt in promoting cell survival, and have led to the identification of apoptosis-promoting proteins as targets of inhibitory Akt phosphorylations (e.g. BAD, Caspase 9, FKHRL1) [11,17,22,23,49–53] (for review see [54]). Perhaps of particular interest is the role that Akt plays in blocking anoikis. Anoikis is a term reserved for the apoptosis induced upon cell detachment [55]. This form of apoptotic death might be expected to play an important role in preventing transformed tumor epithelial cells from becoming metastatic or invasive. Expression of activated RAS in certain cells leads to the inhibition of anoikis. In this system inhibition of anoikis is linked to activation of Akt kinase activity and can be blocked by dominant negative forms of Akt [56]. These data

suggest that epithelial cell survival under detached conditions might be critically dependent upon activation of Akt.

The anti-apoptotic role of Akt suggests that PTEN loss might influence cell survival. When tested PTEN−/− fibroblasts manifest an abrogated apoptotic response to stimuli, such as UV irradiation, TNFα, and hyperosmolarity, a defect that is restored with reintroduction of PTEN [35]. Likewise, in certain PTEN null tumor cells re-expression of PTEN induces apoptosis. Finally, in U251 glioblastoma cells PTEN re-expression restores anoikis to this cell type [47,57,58]. As predicted, the role of PTEN in inducing apoptotic cell death is dependent on or at least redundant to inhibition of Akt activity [35,47]. The role of PTEN in regulating apoptosis is also supported by studies demonstrating defects in Annexin V staining of B cells and macrophages in the bone marrow of *PTEN*+/− mice. These data in particular suggest that, in vivo loss of PTEN is associated with an apoptotic defect in these cell [59]. These particular PTEN +/− mice are markedly predisposed to lymphoid hyperplasia and a defect in apoptosis, rather than proliferation, may account for the lymphoid hyperplasia [59].

Thus, PTEN can act to restrain cell growth by regulating apoptosis or by regulating cell-cycle progression, yet, at the molecular level, both of these activities appear to require inhibition of the PI3K/Akt pathway. It is also clear that certain tumor cell lines and certain murine tissues appear to differ with respect whether the PI3K/PTEN/Akt pathway is primarily involved in regulating survival or proliferation. Whether these contextual differences are linked to cell-type differences or to differences among tumors is not yet clear. Among the many possibilities are that tumors or specific cell-types have divergent expression of downstream targets of Akt and consequently utilize Akt signaling for different cellular functions. Alternatively, and perhaps more likely, tumor cells may have sustained additional mutational events that abrogate PTEN induced apoptosis, for example.

3.4. PTEN and Caenorhabditis elegans

PTEN function has been linked to a PI3K pathway in *C. elegans* that governs aging. Under certain conditions, *C. elegans* worms enter an arrested developmental state known as a dauer state. In this state, feeding and metabolism are suppressed, and the animals demonstrate a prolonged life-span. Genetic analysis of this pathway has led to the characterization of mutant worms that are defective in dauer regulation (dauer affected or daf). Among the first characterized of these mutants was the daf-2 allele that encodes a insulin receptor family member. Subsequently a PI3K homologue (AGE-I) has been identified. Disruption of this gene leads to a constitutive dauer state. *Daf-18* is an allele that bypasses the need for AGE-I in *C. elegans* (for review see [60]). *Daf-18* is now known to encode the *C. elegans* homolog of PTEN [61–64]. Thus, loss of PTEN function rescues the loss of PI3K phenotype. This pathway in *C. elegans* also includes Akt-1, Akt-2, PDK1 and daf-16 a forkhead-related transcription factor. These elegant genetic data have laid out a road map for this pathway that is clearly conserved in mammalian cells. Whether the genetics of aging and metabolism in *C. elegans* will exactly parallel the genetics of this pathway in human tumors remains to be seen, but mounting evidence suggests that this may be the cancer pathway as well.

3.5. The PI3K/PTEN/Akt pathway and cancer

The study of pathways that regulate the transformation of a normal cell into one endowed with the properties associated with malignant growth has repeatedly demonstrated that such pathways are targeted for alterations at different nodal points. In the RB pathway, deregulation of the pathway can be achieved through inactivation of p16/INK4A, through amplification of cyclin D1, through amplification or expression of activated forms of cdk4, and through direct loss or mutation of the RB gene (for review see [65,66]). Furthermore, critical pathways are often targeted by DNA and RNA tumor viruses. The RB and p53 pathways are both targeted by-products of adenovirus, human papilloma virus, and the simian SV40 virus. In this regard, the PI3K/PTEN/Akt pathway is no different. As shown in Table 1, this pathway is under siege by viral oncoproteins and by tumor-associated mutational events.

Table 1
Downstream targets of Akt

BAD	Prevents binding to Bcl-X_L	Datta et al. [23]
Caspase-9	Inhibits protease activity	Cardone et al. [24]
GSK3	Inhibits kinase activity	Cross et al. [18] Dudek et al. [53] Vanhesebroeck et al. (1997)
Forkhead:	Prevents nuclear localization	
FKHRL1		Brunet et al.[17]
AFX		Kops et al. [21]
FKHR		Tang et al. [28] Nakae et al. [121] Guo et al. [19]
CREB	Stimulation of transcriptional activity	Keyong et al. [122]
eNOS	Enhances enzymatic activity	Dimmeler et al. [123] Fulton et al. [124] Michell et al. [125]
IKKα	Prevents binding to NF-κB	Ozes et al. [29] Romashkova and Makarov [30] Kane et al. [31]

In this pathway, upstream of PTEN and of PI3K lie a number of growth and survival factors including PDGF, EGF, insulin, IGF-1, IL-3, and CSF-1, all of which signal, at least in part, through activation of PI3K activity. Both the growth factors themselves and receptors have been targeted subverted for use by retroviruses (e.g. v-sis, v-fms) (see Table 2). Likewise, in human tumors, amplification of growth factor receptors, such as EGF-R, is amply documented. In addition, recent data have linked the risk of developing prostate cancer to the serum level of IGF-1 growth factor in men. Non-receptor tyrosine kinases, such as src, abl, and yes, can act to deregulate this pathway. In particular, it appears that the BCR-ABL fusion protein can activate PI3K and that this activation is required for transformation by BCR-ABL [67].

The PI3K complex, itself, is targeted by three different oncoproteins. Polyoma Middle T antigen activates PI3K by acting as a decoy receptor. Middle T localizes to the plasma membrane via its C-terminus and recruits non-receptor tyrosine kinases, such as src or yes. These kinases phosphorylate Middle T creating docking sites for the p85 subunit, thus, allowing recruitment of PI3K activity to sites of Middle T localized in the plasma membrane. Phosphorylation of p85 then allows recruitment and activation of PI3K. The regulatory subunit p85 has been described as an oncogene in a radiation-induced murine lymphoma and the catalytic subunit p110 has been found as the product of a retroviral oncogene *v-pi3k* [68,69]. Recently, *PIK3CA*, the gene for the p110α catalytic subunit, was found to be amplified in a subset of ovarian tumors [70].

As will be described in greater detail below, *PTEN* is altered through mutation and deletion. In addition, there has been some evidence that methylation might alter PTEN levels. To date, no viral oncoprotein has been implicated in inactivation of PTEN function.

Table 2
Disruption of the PI3K/PTEN/Akt pathway in tumors and by viral oncoproteins

Gene product	Viral interventions	Tumor-derived alterations
Growth factors (examples)		
PDGF	v-sis	
IGF-1		increased risk of CaP*
IL-3		translocations
Growth factor receptors		
PDGF-R		amplification
IGF-1R		
IL-3R		
CSF-1	v-fms	
EGF-R	v-erbA	amplification
Non-receptor tyrosine kinases		
Src	v-src	
Abl	v-abl	BCR-Abl translocation
fyn	v-fyn	
yes	v-yes	
Ras		activating mutations
PI3K (p85 and p110)	middle T antigen	amplification of p110 gene
	v-pi3k	murine lymphoma oncogene
Akt-1	v-akt	none reported
Akt-2	none	amplification
Akt-3	none	overexpression
PTEN	none	mutation, deletion
FKHR	none	translocations

Downstream targets of the active PI3K enzyme include *Akt-1* which, as stated before, was first identified as the viral oncogene *v-akt*. In human tumors neither activating mutations nor amplifications of *Akt-1* have been identified, whereas amplification of *Akt-2* has been reported in ovarian, breast, and pancreatic cancers and cell lines [71–73]. Finally, DU145 prostate cells, that retain a wild-type PTEN allele, appear to contain increased levels of Akt-3, while two PTEN null cell lines, LNCaP and PC-3, do not [74]. This last bit of data perhaps suggests that in cells where PTEN is intact, alternative mechanisms for deregulating the pathway might exist.

Downstream of Akt, Forkhead related transcription factors appear to be important targets of inhibitory phosphorylations. FKHRL1 and FKHR can promote apoptosis, perhaps, in part, through activation of the FasL gene promoter [17,28]. In the presence of activated Akt, the pro-apoptotic function of these proteins is, in effect, silenced. These data suggest that these Forkhead proteins might normally function as suppressors of tumor cell growth. FKHR is involved in two reciprocal translocations found in alveolar rhabdomyosarcoma. These translocations, t(2;13) and t(1;13) create fusions of either PAX3 or PAX7 with FKHR [75,76]. Current research suggests that these fusions create an activated form of PAX3 or PAX7 [77–80]. However, the occurrence of FKHR as the common gene in the two different translocations might be more than coincidental and leads one to wonder whether these translocation give rise to fusion proteins that interfere with FKHR function. If so, one possibility is that PAX3 and PAX7 provide the tissue-restricted expression of a dominantly interfering FKHR truncation.

Finally, 4EBP-1 functions by antagonizing translation initiation factor, eIF4F. This protein is transforming in rodent cells and is overexpressed in human breast and head and neck tumors (for review see [81]), though it has not been reported as a direct target of activating amplification or mutation.

Based upon these observations, it is apparent that the PI3K/PTEN/Akt pathway is frequently altered in human cancers. It is quite possible that, like pRB and p53, inactivation of this pathway, by some mechanism, is a nearly universal phenomenon in human tumors.

4. PTEN: an antagonist of cell spreading and FAK

4.1. PTEN is a protein phosphatase

Are there other PTEN functions and if so, are they required for the ability of PTEN to suppress tumor formation? As stated earlier, PTEN can dephosphorylate protein substrates. In particular PTEN can, in vitro and when overexpressed in cells, dephosphorylate focal adhesion kinase (FAK) and expression of PTEN in *PTEN*+/+ or *PTEN*−/− cells results in a decrease in cell spreading and in cell motility. As PI3K activity has been found in association with FAK, and PI3K can regulate cell ruffling and aspects of cytoskeletal function, the aforementioned effects of PTEN could still result from antagonism of the PI3K pathway. However, a PTEN mutant (PTEN;G129E) that lack lipids phosphatase activity and lacks the ability to induce a G1 arrest, or apoptosis, nonetheless retains protein phosphatase activity and retains the ability to inhibit cell spreading and motility. Thus, this inhibitory activity is clearly a function of PTEN that is independent of PI3K regulation [82].

4.2. What is the role of PTEN protein phosphatase activity in tumor suppression?

PTEN inhibition of cell spreading and cell motility is clearly not *sufficient* for the suppression of the Cowden disease phenotype nor of some somatic tumors. This conclusion is based on the Cowden-derived mutation (G129E) and on tumor derived mutations that, as described above, lack lipid phosphatase, but retain protein phosphatase activity. Thus, in these situations, the preservation of the protein phosphatase activity and, by extension, PTEN inhibition of cell spreading or motility does not suppress the development of human disease. These data, however, do not preclude a role for the protein phosphatase activity in certain aspects of PTEN-mediated tumor suppression. Currently, there is no data to address the question as to whether the protein phosphatase activity, while not sufficient, might be necessary for some aspects of tumor suppression by PTEN. Specifically, to date, no mutant that retains only lipid phosphatase activity has been reported. Such a mutant would allow one to ask whether

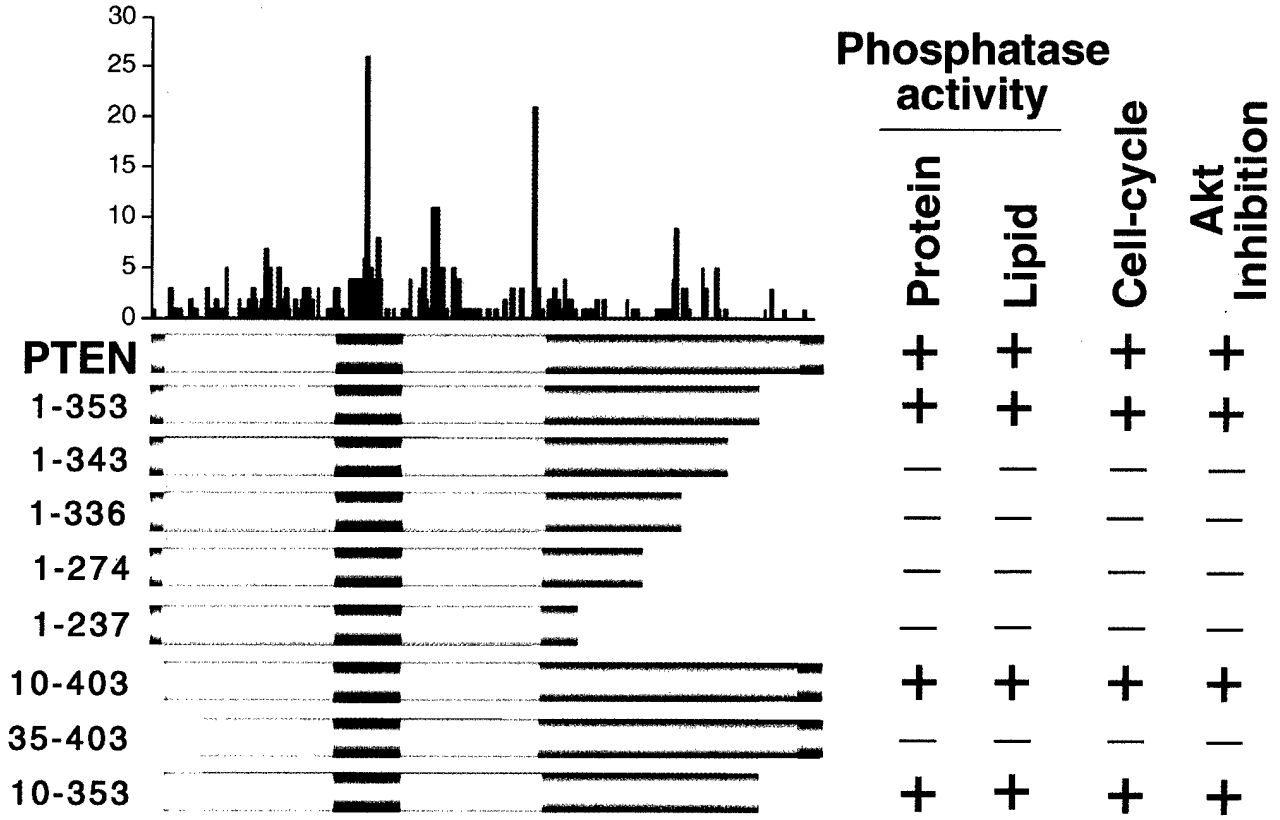

Fig. 2. Mutations and structure–function analysis of PTEN. Shown schematically are the homology domains of PTEN. The light green region represents the region of homology to dual-specificity phosphatases (VHR homology domain). The dark gray box in the middle represents the core phosphatase motif containing the catalytic cysteine. The light gray area at the C-terminus represents a PDZ binding motif. C-terminal and N-terminal truncation are shown along with the activity in the protein and lipid phosphatase assays and in cell-cycle arrest and Akt inhibition assays. The large gray background box represents the minimal functional PTEN phosphatase domain. The histogram represents the frequency of mutation along the *y*-axis and the position of the first altered codon along the *x*-axis. For simplification, the histogram reflects the frequency for each pair of amino acids in PTEN.

PTEN lipid phosphatase activity is sufficient for tumor suppression and, conversely, whether the protein phosphatase activity was a necessary function.

Another possibility that has not been addressed is whether the PTEN protein phosphatase activity plays an independent role in suppressing metastasis. The PTEN;G129E mutant does not suppress tumor cell growth in soft-agar, nor does it induce cell-cycle arrest, but it does retain cell spreading inhibitory activity. Thus, this mutant could be tested for metastasis suppression in model systems [37–39,83].

5. PTEN: structure–function correlations

PTEN somatic mutations are found in certain hotspots that lie within the domain that has homology to dual-specificity phosphatases, such as VHR (for simplicity referred to as the VHR homology domain) (see Fig. 2). Mutations are also found throughout the rest of the coding region. Our group has mapped the regions of PTEN required for protein and lipid phosphatase activity and found that these activities require PTEN residues 10–353 [83]. This is a large portion of the protein and, thus, nearly all mutations fall within the domain required for phosphatase activity. Moreover, missense mutations outside of the VHR homology domain, but within the PTEN phosphatase domain, inactivate catalytic activity. This domain, when expressed in cells, is sufficient to inhibit Akt and sufficient for the induction of a cell-cycle block suggesting that the PTEN phosphatase activity is sufficient for regulation of the PI3K pathway. Thus, most tumor-derived mutations will inac-

tivate these functions of PTEN. The C-terminal 50 residues are not required for phosphatase activity. Whether the phosphatase domain is regulated or modified by flanking C-terminal residues is under investigation.

6. PTEN mutations

6.1. Germline mutations in man

Germline mutations of PTEN are associated with the inheritance of a group of hamartoma-macrocephaly diseases that includes Cowden disease (CD) and Bannayan–Ruvalcaba–Rily syndrome (BRR). Recent data suggests that these groupings primarily reflect phenotypic variation of a single disease entity [84]. Phenotypic characteristics can include mucocutaneous lesions (e.g. trichilemmonmas), macrocephaly, mental retardation, gastrointestinal hamartomas, lipomas, thryoid adenomas, fibrocytstic disease of the breast, and cerebellar dysplastic gangliocytoma (Lhermitte–Duclos disease) (for review see [85]). In addition, Cowden disease is associated with an increased predisposition to breast and thyroid malignancies. Affected females have a lifetime risk of breast cancer that is 2–5 times greater than unaffected women and tumors present, on average, 10 years earlier [85].

Germline point mutations of PTEN and the inheritance of such mutations along with the disease phenotype have been demonstrated by several groups [86–89]. The variation in the phenotype that is associated with inheritance of a mutated copy of PTEN has led to speculation that certain types of PTEN mutation might be associated with different phenotypes. For example, as stated above, certain PTEN mutations are associated with the preservation of protein phosphatase activity. Differences in PTEN genotypes, however, do not appear to account for the differences in phenotypes. First, identical mutations have been associated with two different phenotypic groupings, Cowden disease and Bannayan–Ruvalcaba'Rily syndrome. Second, all of the known Cowden disease germline mutations map to the minimal functional PTEN phosphatase domain, suggesting that they should all interfere with phosphatase activity [83]. Third, multiple pedigrees have been described in which both syndromes are found [84]. Thus, it would appear that the phenotypic differences likely arise as a consequence of differences in the rate of PTEN loss, or as a result of allelic differences in other disease-modifying genes, as yet unidentified. Whether allelic differences in genes in the PI3K/PTEN/Akt pathway might modify (enhance or mitigate) the effects of PTEN loss and, therefore, account for the phenotypic variation in the PTEN-related syndromes is an intriguing possibility.

6.2. Germline mutations in mouse

Disruption of the *PTEN* gene in the murine germline has been reported by three groups. *PTEN*−/− mice generated by eliminating exons 3–5 are inviable with resorbed embryos found at day 9.5 Heterozygous littermates are viable and are predisposed to the development of T-cell lymphoma or leukemia, teratocarcinoma and microscopic hamartomas of the colon. Radiation of these mice induces the development of thymic lymphoma with a shortened latency suggesting that *PTEN* loss might be accelerated by radiation. In addition, a single reported mouse developed prostate cancer [90]. *PTEN*−/− mice generated through a deletion of exons 4–6 are non-viable at day 7.5. Heterozygous littermates are predisposed to carcinomas of the colon and thyroid as well as teratocarcinoma and gonadal stromal tumors. These mice have hyperplastic changes in the prostate, gastrointestinal tract, thyroid, and skin [48]. Finally, PTEN−/− mice derived from the deletions of exon 4 and 5 are non-viable, dying between days 6.5 and 9.5. Heterozygotes are prone to follicular or papillary neoplasia of the thyroid, teratomas, lymphoma, and leukemia. In addition, these mice develop prostate, thyroid, and endometrial hyperplasia, and adenomas of the colon, marked lymph node enlargement, and lymphoid intestinal polyps. Interestingly, the lymphoid hyperplasia appears to be more pronounced in female mice, suggesting that endocrine factors or other genetic loci might modify the PTEN phenotype [59].

These data support the role of PTEN as a tumor suppressor gene in mice. In particular, loss of *PTEN* appears to predispose to a variety of proliferative precursor lesions, suggesting that PTEN functions primarily as a 'gatekeeper' tumor suppressor.

6.3. PTEN mutations in somatic tumors are a late or an early event

6.3.1. Late event

PTEN mutations have been described in a wide variety of cancers. In high grade malignant astrocytoma, known as glioblastoma multiforme (GBM), PTEN mutations are found in 20–30% of primary tumors, while PTEN mutations are rare to absent in lower grade astrocytic tumors, such as anaplastic astrocytomas [91–97]. In GBM, EGFR amplification is common, but does not appear to correlate either positively or negatively with PTEN loss, suggesting that the EGFR and PTEN pathways are not redundant [91,92,97].

PTEN mutations are found in 12–15% of primary prostate tumors and in 60% of xenografts and lines derived from primary and metastatic tumors [98–100]. In addition, as many as 60% of patients with metastatic lesions are found to have a focus of prostate cancer in which PTEN is mutated [101]. PTEN protein is absent in 20% of primary tumors when studied by immunohistochemistry and in this series of tumors loss of PTEN was correlated with higher grade and higher stage tumors [102]. Thus, as in GBM, PTEN loss is associated with a more aggressive prostate tumor. Whether PTEN loss can be used as a predictor of outcome is not known.

6.3.2. Early event

Whereas mutation of PTEN appears to correlate with advanced grade in gliomas and prostate tumors, PTEN mutations can be found in the earlier stages of endometrial cancer. Specifically, PTEN mutations are frequently found in endometrioid sub-type as opposed to the serous histological sub-type of endometrial cancer [103,104]. The former, but not the latter, arises from a proliferative precursor known as endometrial hyperplasia. When PTEN mutations are sought in this precursor lesion, they are found in 20–27% of samples [105,106]. While PTEN mutations in ovarian cancers are not common, they were found in grade I endometrioid ovarian cancers [103,107,108]. Thus, in these two diseases, when PTEN loss occurs, it appears to be a relatively 'early' event.

6.4. PTEN mutation in other tumors

PTEN mutations have been described in a number of other tumors, including melanoma, non-small and small cell lung cancer, thyroid cancer, lymphoma, leukemia, squamous cell cancers of the head and neck, and bladder and renal cell carcinoma [94,109–118]. Whether PTEN loss is associated with more advanced tumors in these diseases is not known.

6.5. What is the relationship between 10q23 loss and PTEN mutation?

There are a number of tumors in which the rate of loss of heterozygosity at the 10q23 locus is considerably higher than the rate of loss or mutation of the second PTEN allele. While this could result from failure to detect mutations or failure to consider epigenetic means of inactivation, there appears to be a persistent discrepancy with respect to this locus. For example, in prostate cancer, glioblastoma, renal, and bladder cancers, the rates of 10q23 loss clearly exceed the rate of detection of PTEN mutations. Nowhere is this more clearly demonstrated than in primary, invasive ductal breast carcinomas where 10q23 loss can approach 40%. In such tumors, however, PTEN mutation has been demonstrated in a relatively small subset (4 of 68 tumors) [119,120]. These data suggest the possibility that other tumor suppressor genes lie within this locus. On the other hand, patients afflicted with Cowden disease are at substantially greater risk of developing breast cancer than unaffected individuals. Thus, PTEN can clearly play a tumor suppressor role in the mammary gland epithelium. Studies of the PTEN+/+, +/− and −/− fibroblasts suggest that there is a *PTEN* gene-dosage effect with respect to Akt deregulation [36]. Furthermore, heterozygous loss of PTEN in the mouse thyroid and prostate leads to an increase in the proliferative index of these tissues [48]. Taken together, these data raise the possibility that heterozygous loss of PTEN, alone, might play a role in the neoplastic process.

7. Summary

The *PTEN* tumor suppressor and the PI3K/PTEN/Akt pathway appear to be critical regulators of apoptosis and cell-cycle progression. It is likely that, with time, the frequency of mutations and the spectrum of tumors found to have a deregulating alteration in the pathway will increase. Many of the functional members of this pathway that remain and are activated in the absence of PTEN encode kinases and, as such, are attractive targets for anticancer therapy. A critical question that remains is whether cancer cells that are deregulated for this pathway are more or less sensitive to pathway-inhibiting interventions than their normal counterparts. If so, inhibitors of this pathway could find widespread clinical utility.

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