Negative Regulation of PKB/Akt-Dependent Cell Survival by the Tumor Suppressor PTEN

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Summary

PTEN is a tumor suppressor with sequence homology to protein tyrosine phosphatases and the cytoskeletal protein tensin. mPTEN-mutant mouse embryos display regions of increased proliferation. In contrast, mPTEN-deficient immortalized mouse embryonic fibroblasts exhibit decreased sensitivity to cell death in response to a number of apoptotic stimuli, accompanied by constitutively elevated activity and phosphorylation of protein kinase B/Akt, a crucial regulator of cell survival. Expression of exogenous PTEN in mutant cells restores both their sensitivity to agonist-induced apoptosis and normal pattern of PKB/Akt phosphorylation. Furthermore, PTEN negatively regulates intracellular levels of phosphatidylinositol (3,4,5) trisphosphate in cells and dephosphorylates it in vitro. Our results show that PTEN may exert its role as a tumor suppressor by negatively regulating the PI3'K/PKB/Akt signaling pathway.

Introduction

Loss of heterozygosity on human chromosome 10 is the most common genetic alteration in the development of malignant gliomas (Bigner et al., 1998). Recent identification of PTEN (also known as MMAC1 and TEP1) (Li and Sun, 1997; Li et al., 1997; Steck et al., 1997) as the candidate tumor suppressor gene located on chromosome 10 initiated a number of studies that have demonstrated a significant rate of mutations of the PTEN gene not only in gliomas (Rasheed et al., 1997) but also in intracellular levels of PI(3,4,5)P3, most likely via direct evidence that a large proportion of tumor-associated PTEN mutations map to the region encoding the phosphatase domain (Rasheed et al., 1997; Marsh et al., 1998). Furthermore, unlike wild-type PTEN, catalytically inactive PTEN is unable to suppress growth and tumorigenicity of PTEN-deficient glioblastoma cells (Furnari et al., 1997).

The N-terminal domain of PTEN shows extensive homology to tensin, a protein associated with the actin cytoskeleton at focal adhesions (Lo et al., 1994). Recent evidence demonstrates that overexpressed PTEN interacts directly with the actin cytoskeleton at focal adhesions. Expression of PTEN in immortalized mouse embryonic fibroblasts decreases its tyrosine phospholipidation. In addition, PTEN was shown to inhibit cell migration, integrin-mediated cell spreading, and formation of focal adhesions (Tamura et al., 1998). Thus, both genetic and biochemical evidence has implicated PTEN in the regulation of several different cellular processes: cell growth, extracellular matrix interactions, and/or cell migration.

To determine the role of the PTEN in normal development and tumorigenesis, we have generated mPTEN-deficient mice by targeted disruption of exons 3 to 5. Homozygous mPTEN-5 mice die by day 9.5 of development and show abnormal patterning and overgrowth of the cephalic and caudal regions (A. S. et al., submitted). Analysis of mPTEN-5 embryos reveals regions of increased proliferation. In contrast, immortalized mouse embryonic fibroblast cell lines from mPTEN-5 homozygous mutant embryos show decreased sensitivity to various apoptotic stimuli, including UV irradiation, heat shock, osmotic stress, and tumor necrosis factor stimulation. mPTEN mutant cells display constitutively elevated activity of PKB/Akt, which can be restored to normal levels by reexpression of mPTEN. Consistent with these findings, an active mutant of PKB/Akt suppresses apoptosis induced by mPTEN overexpression. Our results demonstrate that PTEN negatively regulates intracellular levels of PI(3,4,5)P3, most likely via direct dephosphorylation, providing a mechanism for PTEN action. We propose that PTEN exerts its role as a tumor suppressor by negatively regulating the PI3'K/PKB/Akt signaling pathway.

Results

Enhanced Proliferation in E8.5 mPTEN-5 Mutant Embryos

mPTEN-deficient mice were generated by targeted disruption of exons 3 to 5 of the murine PTEN gene. Homozy-
Figure 1. Extensive Proliferation of mPTEN35 Mutant Embryos In Vitro and In Vivo
(A) Wild-type blastocyst outgrowth after 10 days of culture. The inner cell mass (ICM) has grown extensively and remains coherent.
(B) mPTEN35 mutant blastocyst showing a large ICM that spreads out in the dish.
(C) E7.5 wild-type embryo. Strongly BrdU-positive cells can be seen throughout all embryonic regions. al, allantois; ch, chorion; ee, embryonic ectoderm; m, mesoderm.
(D) E7.5 mPTEN35 mutant embryo showing strong BrdU incorporation and a very thick ectoderm region (arrow).
(E) Cross section through the headfold region of a E8.5 wild-type embryo, showing BrdU staining in the neuroepithelium (arrowhead) and underlying mesenchyme (mes).
(F) Cross section of the headfold of a mPTEN35 mutant embryo. Very strong staining can be seen in the neuroepithelium (arrowhead) and underlying mesenchyme. Bar, 40 μm in (A) and (B); 140 μm in (C)-(F).

gous mPTEN35 mutant mice die by day 9.5 of development (E9.5), and, at E8.5, a typical phenotypic feature of the mutants is dramatic overgrowth in the cephalic and caudal regions (A. S. et al., submitted), suggesting a possible defect in programmed cell death or cellular proliferation. Apoptosis, as measured by TUNEL assay (Gavriel et al., 1992) or DAPI staining, showed no obvious differences between wild-type and mutant embryos (data not shown).

Next, we analyzed the proliferative capacity of mPTEN35 mutant embryos in vitro and in vivo. E3.5 blastocysts cultured in vitro organize into a compact inner cell mass and a surrounding trophoblast (Figure 1A). mPTEN35 mutant blastocysts grow similarly to wild-type littermates for a period of up to 10 days, although they tend to appear more expanded and disorganized relative to normal littermates (Figure 1B). To measure directly the effects of the mPTEN35 mutation on cellular proliferation, we examined the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA during the S phase of the cell cycle. Analysis was performed at E7.5 and E8.5, at times when the mitotic activity in the mouse embryo undergoing organogenesis is high (Snow, 1977). A proliferative index based on the ratio of proliferating cells (BrdU-positive nuclei) to total cell number was calculated. Wild-type embryos showed extensive BrdU labeling, particularly in the ectoderm region (Figure 1C). A total of three wild-type embryos were analyzed, and they showed a proliferative index of 0.7 ± 0.1. mPTEN35 mutant embryos appear more primitive and show very strong BrdU staining in all germ layers (Figure 1D). Three mutant embryos analyzed gave an index of 0.8 ± 0.1. At E8.5, differences in proliferation between wild-type and mutant embryos were apparent. Figure 1E shows BrdU staining in the cephalic region of a E8.5 wild-type embryo. BrdU-positive cells appear scattered throughout the neuroepithelium of the head folds and underlying cephalic mesenchyme. Three wild-type embryos were analyzed at E8.5, and they gave a proliferative index of 0.7 ± 0.05. In contrast, mPTEN35 mutant embryos show BrdU staining throughout the deformed head folds and in the cephalic mesenchyme that is significantly stronger than in wild-type embryos (Figure 1F). Three mutant embryos analyzed gave an index of 0.9 ± 0.03. These values were statistically significantly different (p < 0.05, Student’s t test). Thus, BrdU incorporation is quantitatively increased in mutant embryos, suggesting that enhanced proliferation is involved in the abnormal development of the cephalic region of mPTEN35 mutant embryos. Similarly, increased BrdU incorporation was
To further investigate the role of PTEN in regulation of (GFP) as a control. As measured by fluorescence of blast cell lines from two mPTEN cell lines confirmed the loss of mPTEN expression in Immunoblotting of cell lysates from the established ing that the generated cells are fibroblasts (Figure 2). cell survival. (Strutz et al., 1995) in all three cell lines, demonstrat- confirmed expression of mPTEN in mouse NIH 3T3 cells also (A) Western blot analysis of whole-cell lysates from brain, thymus, and mPTEN+/− and mPTEN−/− cell lines. The positions of molecular weight markers (kDa) are shown on the left. The position of PTEN is indicated by an arrow. (B) RT-PCR analysis of mPTEN+/− and mPTEN−/− cell lines. FSP1 indicates the reactions performed using fibroblast-specific protein 1-specific primers. β2M indicates the reactions performed using β2 microglobulin-specific primers. H2O, water control (no RNA); spleen, total RNA from normal mouse splenocytes; mPTEN+/−, total RNA from mPTEN+/− cells; mPTEN−/−, total RNA from mPTEN−/− cells; mPTEN−/−, total RNA from mPTEN−/− cells. observed in the allantois at the posterior end of the embryo (data not shown).

mPTEN-Deficient Mouse Embryonic Fibroblasts Exhibit Decreased Sensitivity to Apoptosis

To further investigate the role of PTEN in regulation of cellular processes, immortalized mouse embryonic fibroblast cell lines from two mPTEN−/− and mPTEN+/−, respectively) and one heterozygous mutant embryo (mPTEN+/−) were generated. Reverse transcription–polymerase chain reaction (RT-PCR) using RNA from generated cells shows the expression of fibroblast-specific protein 1 (FSP1) (Strutz et al., 1995) in all three cell lines, demonstrating that the generated cells are fibroblasts (Figure 2). Immunoblotting of cell lysates from the established cell lines confirmed the loss of mPTEN expression in both mPTEN+/− and mPTEN−/− cell lines, whereas mPTEN−/− expressed a protein of the molecular mass of approximately 55 kDa (Figure 2). The apparent molecular mass of mPTEN is consistent with a major immunoreactive species identified in brain, thymus, and NIH3T3 cells (Figure 2) and of recombinant mPTEN (data not shown). Both mPTEN−/− cell lines showed no morphological differences in comparison to their heterozygous counterpart (data not shown).

Surprisingly, we observed no significant difference in the rate of proliferation between the mPTEN homo- and heterozygous cells (data not shown). We next examined the rate of agonist-induced cell death in heterozygous and mPTEN-deficient cells. As measured by viability dye staining, more than 80% of mPTEN+/− and mPTEN−/− cells were viable 24 hr following treatment with UV irradiation (780 J/m²), sorbitol (0.4 mM), TNFα (10 ng/ml) cycloheximide (5 μg/ml), anisomycin (10 μg/ml), or 30 min of heat shock at 42.5°C (Figure 3A). In contrast, these same treatments resulted in extensive cell death in mPTEN+/− cells. Strikingly, even 1 day after a 2 hr heat shock, almost 40% of mPTEN−/− cells were still viable (Figure 3A). Similar results were obtained using early and late markers of programmed cell death, annexin V, and propidium iodide, respectively, suggesting that the mPTEN−/− cells were dying from apoptosis (data not shown). To further test this possibility, 8 hr after treatment, nuclear morphology of mPTEN+/− and mPTEN−/− cells was examined. Nuclei of mPTEN−/− cells display chromatin condensation indicative of apoptosis, whereas nuclei of mPTEN+/− appear normal (Figure 3B). Prolonged monitoring of mPTEN−/− cells indicates that they eventually die in response to UV irradiation and sorbitol treatment (Figure 3C), as well as other apoptotic stimuli (data not shown), although at a significantly reduced rate in comparison to the mPTEN+/− cells. A similar decrease in sensitivity to apoptosis was observed in mPTEN-deficient ES cells (data not shown). Thus, reduced sensitivity of mPTEN-negative cells to apoptosis suggests that PTEN might exert its function as a tumor suppressor by negatively regulating cell survival.

Expression of Exogenous mPTEN in mPTEN-Deficient Cells Restores Sensitivity to Apoptosis

To further investigate whether resistance to apoptosis in mPTEN−/− cells was a result of the loss of PTEN function, mPTEN was expressed in all three cell lines using recombinant retrovirus carrying an HA-tagged copy of wild-type mPTEN, or green fluorescence protein (GFP) as a control. As measured by fluorescence of control cells, the efficiency of retroviral infection was higher than 70% in all three cell lines (data not shown). In contrast to GFP, expression of mPTEN in mPTEN-deficient cell lines restored normal apoptosis in response to all stimuli tested (Figure 4). Retrovirus-mediated expression of mPTEN in mouse NIH 3T3 cells also increased their sensitivity to cell death (data not shown), confirming the role of PTEN as a negative regulator of cell survival.

Increased Activity of Cell Survival Kinase PKB/Akt in PTEN Mutant Cells

One of the key regulatory molecules involved in the regulation of cell survival is protein kinase B/Akt (PKB/Akt). PKB/Akt is implicated in protection from apoptosis in response to several growth factors and cytokines, and it protects cells from apoptosis induced by withdrawal of survival signals, c-Myc overexpression (Kauffmann-Zeh et al., 1997;
Figure 4. Expression of PTEN Restores Susceptibility of PTEN-Deficient Cells to Agonist-Induced Apoptosis

Two days following infection with retroviruses carrying PTEN (hatched bars) or GFP (open bars), cells were treated as indicated and analyzed for viability as in Figure 3. Viability is expressed normalized to the untreated control (5% FCS). Solid bars correspond to noninfected cells. The individual cell lines and cell treatments, respectively, are indicated at the bottom. The presented data are from at least three independent experiments. Error bars represent standard error of the mean expressed in percentage.

(Alessi et al., 1997; Stokoe et al., 1997). Using an antibody specific for PKB/Akt phosphorylated at Ser-473 and kinase assays of immunoprecipitated PKB/Akt proteins, we tested the phosphorylation status and activity of PKB/Akt in our cells (Figure 5A). Under resting (5% FCS) or serum-starvation (0.5% FCS) conditions, the levels of PKB phosphorylation were elevated in both mPTEN<sup>−/−</sup> cells in comparison to mPTEN<sup>+/−</sup> cells. Hyperphosphorylation of PKB/Akt was accompanied by increased activity of PKB/Akt toward a previously characterized PKB/Akt-specific peptide substrate (Cross et al., 1995) (Figure 5A). Stimulation of serum-starved cells with platelet-derived growth factor (PDGF) (50 ng/ml) resulted in similar increase in phosphorylation and activity of PKB/Akt in all three cell lines, demonstrating that the pathway leading to PKB/Akt activation was intact in all cell lines. In addition, basal activity and PDGF-induced activation of mitogen-activated protein kinases Erk1 and Erk2 appeared normal in all three cell lines (data not shown). Pretreatment of serum-starved cells with the specific inhibitor of PI3K, wortmannin (Figure 5A), or LY294002 (data not shown) completely eliminated basal as well as PDGF-dependent increase in PKB/Akt phosphorylation/activity in all cell lines. This result indicates that PI3′K is responsible for both increased PKB phosphorylation/activity in nontreated mPTEN<sup>−/−</sup> cells and activation following PDGF stimulation in all three cell lines. The reduced basal phosphorylation of PKB/Akt (Figure 5B) and activity (data not shown) in mPTEN<sup>−/−</sup> cells could be restored by retroviral expression of mPTEN in these cells, demonstrating a functional interaction between mPTEN and PKB/Akt and an active role of mPTEN in negative regulation of PKB/Akt phosphorylation and activity. In support of such a role is the observation of PKB/Akt hyperphosphorylation in T cell lymphomas that frequently develop in mPTEN heterozygous mice and are associated with the loss of heterozygocity at the mPTEN locus (A. S. et al., submitted).
Figure 5. Negative Regulation of PI3 K/PKB/Akt Signaling Pathway by PTEN

(A) Increased phosphorylation and activity of PKB/Akt in PTEN-deficient cells. (Top) Western blot analysis of cell lysates from cells treated as indicated. 0.5% FCS indicates incubation of cells in 0.5% FCS for 24 hr prior to treatment. PDGF indicates treatment with PDGF (50 ng/ml) for 15 min. W indicates pretreatment of cells with wortmannin (100 nM) for 20 min prior to addition of PDGF. Antibodies used in Western blotting are indicated on the left. Comparable results were obtained from at least four independent experiments. (Bottom) PKB kinase activity in mPTEN−/− and mPTEN+/− cell lines. PKB proteins were immunoprecipitated from cells treated as indicated (see above) and analyzed for incorporation of 32P into a previously characterized peptide substrate (see Experimental Procedures). The data are presented normalized to the activity of PKB/Akt from untreated (5% FCS) mPTEN+/- cells. The presented data are from at least three independent experiments. Error bars represent standard error of the mean expressed in percentage.

(B) Expression of PTEN reduces basal phosphorylation of PKB/Akt in mPTEN−/− cells. Forty-eight hours after infection with the retrovirus expressing HA-PTEN, cells were treated as indicated on top (see [A]). Antibodies used in Western blotting are indicated on the left. Comparable results were obtained from at least three independent experiments.

A Constitutively Active Mutant of PKB/Akt Rescues Cells from PTEN-Induced Apoptosis

Expression of mPTEN using transient transfection of NIH 3T3 cells resulted in a decrease in the number of surviving transfected cells in comparison to the mock-transfected controls, indicating that high levels of mPTEN expression can result in cell death (Figure 6). Using a cotransfection approach, we examined the ability of wild-type or mutant PKB/Akt to alter mPTEN-induced cell death in NIH 3T3 cells 48 hr after transfection. Expression of wild-type PKB/Akt had no influence on mPTEN-mediated cell death (Figure 6). However, coexpression of PKB-DD, a constitutively active PKB/Akt mutant in which Thr-308 and Ser-473 have been changed to aspartate to mimic phosphorylation (Welch et al., 1998; Jing Jin and James R. Woodgett, personal communication), with mPTEN increases the number of surviving transfected cells to the levels comparable to mock-transfected cells (Figure 6). Thus, expression of constitutively active PKB/Akt rescues transfected cells from mPTEN-induced apoptosis and indicates a potential function of mPTEN upstream of PKB/Akt.

PKB-AAA is a dominant-negative PKB/Akt mutant in which both phosphorylation sites and the lysine in the ATP-binding pocket have been mutated to alanine residues, respectively, resulting in a complete loss of kinase activity (Meier et al., 1997; Stephens et al., 1998; Jing Jin and James R. Woodgett, personal communication). Expression of PKB-AAA induced apoptosis in NIH 3T3 cells comparable to the levels achieved by mPTEN transfection alone (Figure 6). Significantly, coexpression of PKB-AAA and mPTEN did not result in an additive effect on cell death (Figure 6), suggesting that PKB and mPTEN act in the same signaling pathway.

The role of mPTEN in PKB/Akt regulation is further supported by the effect of mPTEN on gag-PKB-mediated cell survival. gag-PKB is the oncogenic form of PKB/Akt that was identified as an oncogene carried by AKT8, the acute transforming virus from a rodent T cell lymphoma (AKR) (Bellacosa et al., 1991). The myristyl group of viral gag protein serves to bring gag-PKB fusion protein to the cell membrane, where it becomes activated by upstream kinases (Ahmed et al., 1993; Kennedy...
et al., 1997; Alessi and Cohen 1998). In our studies, transfection of gag-PKB into NIH 3T3 cells resulted in an increase in the number of surviving cells, possibly due to the ability of gag-PKB to protect from transfection-induced death (Figure 6). However, cotransfection of mPTEN with gag-PKB resulted in a decrease in the number of transfected cells comparable to that induced by mPTEN alone (Figure 6). Considering that gag-PKB requires phosphorylation of Thr-308 and Ser-473 for activity (Kennedy et al., 1997), mPTEN is most likely negatively regulating the phosphorylation of both PKB and gag-PKB, consistent with our previous findings (Figure 6).

Phosphatidylinositol (3,4,5)P₃ Is a Direct Substrate of PTEN
The activity of PI3 K under normal or PDGF-stimulation conditions was identical in mPTEN⁻/⁻ and mPTEN⁻/⁻ cells (data not shown), suggesting that mPTEN was affecting the PKB/Akt phosphorylation and activity by acting downstream of PI3 K. Treatment with wortmannin, a specific inhibitor of PI3 K, sensitized mPTEN⁻/⁻ cells to UV irradiation- and sorbitol treatment-induced cell death (Figure 7A), demonstrating that an immediate product of PI3 K activity was responsible for the altered sensitivity of mPTEN⁻/⁻ cells to apoptosis. Furthermore, constitutively active PKB/Akt rescues transfected cells from mPTEN-induced apoptosis, implying a function of mPTEN upstream of PKB/Akt.

PKB/Akt phosphorylation on both Thr-308 and Ser-473 is directly dependent on the levels of intracellular phosphatidylinositol(3,4,5)P₃, the key product of PI3 K activity (Alessi et al., 1996, 1997; M. Delcommene et al., submitted). In mPTEN⁻/⁻ cells, PKB/Akt is hyperphosphorylated under resting conditions (5% FCS) (Figure 5A). Under those conditions, mPTEN⁻/⁻ cells exhibit elevated levels of intracellular PI(3,4,5)P₃ in comparison to their heterozygous counterpart (Figure 7B), demonstrating a direct role of mPTEN in dephosphorylation of PI(3,4,5)P₃. Furthermore, bacterially expressed mPTEN dephosphorylates the D3 position of PI(3,4,5)P₃ in vitro (Figure 7C). Thus, PI(3,4,5)P₃ is a physiological substrate of PTEN.

Discussion

Role of PTEN in Proliferation
mPTEN⁻/⁻ mutant embryos die by E9.5 with abnormal patterning and extensive overgrowth of the cephalic and caudal regions. At E7.5 and more dramatically at E8.5, BrdU incorporation is increased in mPTEN⁻/⁻ embryos is consistent with the ability of ectopic PTEN to suppress cell growth (Furnari et al., 1997; Cheney et al., 1998). Nuclear integrity dye staining of mPTEN⁻/⁻ embryos does not reveal any alterations in apoptosis associated with the mPTEN loss. In contrast, mPTEN-deficient fibroblasts display no increase in proliferation but decreased sensitivity to apoptosis. This apparent discrepancy between the phenotypes of mPTEN⁻/⁻ embryos and cells could be explained by the fact that programmed cell death during early embryogenesis is a rare event (Coucovanis and Martin, 1995), preventing, using...
the available methods, the detection of a potential decrease in apoptosis in mPTEN<sup>−/−</sup> embryos. Hence, hyperproliferation in mPTEN<sup>−/−</sup> embryos could be a consequence of a differentiation defect associated with decreased apoptosis due to mPTEN deficiency. In support of such a view, immunostaining of wild-type mouse embryos demonstrated that tissue distribution of mPTEN coincides with regions where differentiation occurs (data not shown). Alternatively, immortalized embryonic fibroblasts derived from mPTEN<sup>−/−</sup> embryos might not be analogous to the affected cells involved in the overgrowth in the mPTEN<sup>−/−</sup> embryo and therefore might not display an equivalent proliferation defect.

**Role of PTEN in Apoptosis**

Loss of mPTEN in immortalized mouse embryonic fibroblasts results in decreased sensitivity to cell death in response to diverse apoptotic stimuli. The effect of mPTEN loss on apoptosis appears to be pleiotropic considering that mPTEN<sup>−/−</sup> cells no longer die in response to a number of treatments known to affect a variety of different signaling pathways. Such a broad role of mPTEN in regulation of cell survival suggests that mPTEN functions in a general pathway that regulates cell death and survival. The evidence presented here identifies a crucial regulator of cell survival, PKB/Akt, as a major target of mPTEN activity. mPTEN regulates phosphorylation and activity of PKB/Akt in cells. Furthermore, expression of a constitutively active mutant of PKB/Akt rescues cells from PTEN-induced apoptosis. Activation of PKB/Akt has been implicated in protection from apoptosis in response to several growth factors and cytokines, including IGF-1 (Kulik et al., 1997), IL-2 (Ahmed et al., 1997), and NGF (Ulrich et al., 1998). Moreover, elevated PKB/Akt activity protects cells from apoptosis induced by withdrawal of survival signals (reviewed in Downward, 1998), c-myc overexpression (Kauffmann-Zeh et al., 1997), UV irradiation (Kulik et al., 1997), CD95/Fas-induced cell killing (Hausler et al., 1998), and matrix detachment (Kwaja et al., 1997). Decreased sensitivity of mPTEN mutant cells to apoptosis in response to TNFα, sorbitol, and anisomycin suggests that active PKB/Akt also protects cells from death induced by these agents.

Activation of SAPK/J NK, whose function in induction and protection from apoptosis remains controversial (Kyrilakis and Avruch, 1996; Nishina et al., 1997), was normal in our cells in response to UV irradiation, anisomycin, and sorbitol (data not shown), indicating that this pathway is not affected by the loss of mPTEN. Furthermore, TNFα stimulation of all three cell lines resulted in a comparable level of phosphorylation of IkB (data not shown), suggesting that NF-κB activation in our mutant cells was intact and is therefore an unlikely cause of their altered cell death. A recent report (Tamura et al., 1998) has identified direct interaction between focal adhesion kinase (FAK) and PTEN and demonstrated a role of PTEN in regulation of cell migration and spreading. Even though our preliminary evidence does not reveal any differences in matrix attachment between mPTEN<sup>+/+</sup> and <sup>−/−</sup> cells (V. S., unpublished observations), it will be interesting to utilize our mPTEN-deficient cells to further characterize the interaction between FAK and PTEN in vivo.

**PI(3,4,5)P<sub>3</sub> Is a Physiological Substrate of PTEN**

The intact PI3K activity in mPTEN<sup>+/−</sup> cells and the ability of wortmannin to sensitize mPTEN<sup>−/−</sup> cells to agonist-induced apoptosis suggested that the mechanism of mPTEN action might be at the level of the products of PI3K activity. The evidence presented here shows that mPTEN negatively regulates the intracellular levels of PI(3,4,5)P<sub>3</sub>, most likely via direct dephosphorylation, resulting in decreased phosphorylation and activity of PKB/Akt. Consistent with our findings, a recent report (Maehama and Dixon, 1998) has shown that PTEN is capable of dephosphorylating PI(3,4,5)P<sub>3</sub> in vitro and down-regulating its levels in insulin-stimulated 293 cells. Taken together, our results establish PI(3,4,5)P<sub>3</sub> dephosphorylation as a major mechanism of PTEN action. Thus, in the absence of mPTEN, elevated levels of PI(3,4,5)P<sub>3</sub> result in increased PKB/Akt phosphorylation. Conversely, expression of mPTEN negatively regulates PKB/Akt phosphorylation/activity via dephosphorylation of PI(3,4,5)P<sub>3</sub>.

**The Role of PTEN in Tumorigenesis**

PTEN mutations have been identified in a large number of different tumors, indicating that PTEN regulates fundamental cellular processes. Our results provide evidence for such a critical role of PTEN in control of normal cell growth. In certain tumors, loss of PTEN may result in decreased sensitivity of mutant cells to apoptotic stimuli. In other tumors, mutations of PTEN may lead to an increase in proliferative potential. Both survival and proliferative advantages of PTEN-deficient cells would promote cellular overgrowth and tumorigenesis.

PTEN mutations in humans have initially been identified in glioblastomas (Li et al., 1997). Frequent amplification of platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) genes have also been demonstrated in glioblastoma (Chaffanet et al., 1992; Smits and Funa, 1998). Furthermore, the production and cellular response to fibroblast growth factor (FGF) are often elevated in astrocytomas and gliomas (Takahashi et al., 1992). PI3K activation is regulated by the activity of receptor-tyrosine kinases and is potentially up-regulated in cells overexpressing PDGFR and EGFR, as well as in cells exposed to higher than normal levels of FGF. Hypothetically, this would result in increased levels of intracellular PI(3,4,5)P<sub>3</sub> and elevated activity of PKB/Akt, giving the cells a survival advantage. The ability of PKB/Akt to protect cerebral neurons from apoptosis supports such a scenario (Dudek et al., 1997). Moreover, increased expression of PDGFR and EGFR, or FGF overproduction in glioblastoma, could result in increased proliferation. Thus, other genetic alterations in glioblastoma leading to PI3K activation might have similar phenotypic consequences on tumor development as the loss of PTEN function.

v-akt, an oncogenic form of PKB/Akt resulting in increased kinase activity, causes mouse T cell lymphoma...
(AKR) (Staal and Hartley, 1988; Bellacosa et al., 1991). Interestingly, mice heterozygous for mPTEN frequently develop T cell lymphomas that are associated with the loss of heterozygosity at the mPTEN locus (A. S. et al., submitted). These tumors exhibit elevated phosphorylation of PKB/Akt in comparison to normal tissue, consistent with our findings that PTEN negatively regulates PKB/Akt signaling. In humans, overexpression of PKB/Akt has been demonstrated in 15% ovarian (Cheng et al., 1992), 12% of pancreatic (Cheng et al., 1996), and 3% of breast cancers (Bellacosa et al., 1995). Significantly, in addition to mutations shown in other tumors, LOH in the human PTEN locus has been observed in ovarian, pancreatic, and breast tumor specimens (Teng et al., 1997). Such correlation in the rate of LOH of PTEN and up-regulation of PKB/Akt in human cancer, together with the evidence presented here, emphasizes the importance of PTEN/PKB/Akt interaction in tumor progression.

Our results suggest that PTEN belongs to a class of “gatekeeper” tumor suppressor genes together with p53, Rb, and APC. These genes participate in regulation of normal cell growth, and their mutations directly contribute to the neoplastic growth of the cell (Kinzel and Vogelstein, 1998). We propose that negative regulation of P13K/PKB/Akt cell survival signaling pathway is a likely mechanism by which PTEN exerts its tumor suppressor function. Thus, loss of PTEN results in general alteration of the normal cell homeostasis due to increased survival potential, which could subsequently lead to tumorigenesis. Further investigations, including those aimed at elucidating modes of PTEN regulation, will provide valuable insight into the processes of cell transformation and tumorigenesis.

Experimental Procedures

BrdU Labeling of Mouse Embryos

Generation of mPTEN+/− mutant embryonic stem cells and mice will be described elsewhere. BrdU labeling of cells in the 5 phase of the cell cycle was performed according to the protocol described by Hayashi et al. (1988). BrdU (100 mg/g of body weight) was injected intraperitoneally into pregnant females at E7.5 and E8.5 of gestation. Females were sacrificed 1 hr after injection, the uteri removed, and complete decidua or embryos fixed in 4% paraformaldehyde at storage buffer (25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 2 mM DTT) and passed through a Q-Sepharose FF column bed volume of a 0–500 mM NaCl concentration gradient.

Establishment of Immortalized Embryonic Fibroblast Cell Lines

Day 13 heterozygous and homozygous mPTEN+/− embryos (A. S. et al., submitted) were dissected, minced well by scissors, and cultured individually in gelatinized 24-well plates containing Dulbecco modified Eagle’s medium (DMEM), 10% fetal calf serum (FCS), L-glutamine, and β-mercaptoethanol. After 7 days of culture, cells were trypsinized, replated, incubated for 1 hr, and washed three times with PBS to remove nonadherent cells. Adherent cells were continuously cultured for 3 months using the 3T3 protocol (Goldman, 1998). Embryo genotyping was performed by PCR with DNA from the yolk sac, using specific primer sets to detect wild-type and the mutant mPTEN allele, respectively.

RT-PCR

Total RNA was prepared from 5 × 10⁶ cells using TRIZOL reagent as per manufacturer’s protocols (Life Technologies). Intron-spanning oligonucleotide primer pairs specific for mouse β2-microglobulin (sense primer 5'-ATGGCTCTGGCTGGTACCTAG-3' and antisense primer 5'-TCATGATGCTTGATCACATGTCTC-3') or mouse fibroblast-specific protein-1 (sense primer 5'-CAGCGAAGAAGGGTGACAGTTCA-3' and antisense primer 5'-ATGGCCGAAAGCCAGAGTAGG-3') were used to amplify 1 μg of total RNA employing the Titan one-tube RT-PCR system as per manufacturer’s instructions (Boehringer Mannheim/Roche). Reaction products were resolved by 7% polyacrylamide gel electrophoresis and detected by ethidium bromide staining.

Cloning, Expression, and Purification of mPTEN

A cDNA encoding the entire mouse PTEN open reading frame was amplified from mouse brain mRNA by RT-PCR as above using sense primer 5'-GGATCCGACATGACGGCATGACACATCAAAAG-3' and antisense primer 5'-CTCGAGTCGACATTATTTGTATTTGATGAGTGCTG-3', sequenced, and subcloned in-frame with two N-terminal influenza virus hemagglutinin (HA) epitopes into the eukaryotic expression vector pcDNA3.1 (Invitrogen). An EcoRI-Xhol fragment from the resultant pcDNA3.1HAzmPTEN vector, encoding N-terminally HA-tagged mPTEN open reading frame, was ligated into the EcoRI-Sall sites of the retroviral vector pBabeMN-z (a kind gift of Dr. Gary Nolan). Full-length mPTEN cDNA and a fragment encoding a C-terminal portion of mPTEN (amino acids 151–403 of SwissProt O08586) were separately cloned into the thioredoxin-hexahistidine tag (Trx) fusion vector pET32a (Novagen) and transfected into E. coli strain BL21(DE3)pLysS or BL21(DE3), respectively (Strategene, La jolla, CA).

Expression of Trx-mPTEN and Trx-mPTEN(151–403) fusion proteins was induced in bacterial cultures at an OD_{600} of 0.8 by the incubation in the presence of 0.8 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 hr at 37°C or 6 hr at 28°C, respectively. Cells were lysed by sonication in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 20 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, 0.5% Nonidet P-40, and protease inhibitors). The cell lysate was clarified by centrifugation, and supernatants were passed through nickel-nitrilotriacetic acid resin (Qiagen, Valencia, CA). Washed with lysis buffer, and hexahistidine-tagged fusion protein was eluted using an imidazole concentration gradient. Eluted Trx-mPTEN(151–403) protein was dialysed against storage buffer (50 mM Tris-HCl [pH 8.0], 2 mM EDTA, 2 mM DTT, 150 mM NaCl) and concentrated by ultrafiltration.

Eluted Trx-mPTEN fusion protein was dialysed against ion-exchange column equilibration buffer (25 mM Tris-HCl [pH 8.0], 2 mM EDTA, and 2 mM DTT) and passed through a Q-Sepharose FF ion exchange column (Pharmacia Biotech, Inc.) equilibrated in the same buffer. The column was washed with 10 column bed volume of equilibration buffer, and Trx-mPTEN protein was eluted using 20 column bed volume of a 0–500 mM NaCl concentration gradient. The Trx-mPTEN containing fractions were pooled, dialysed against storage buffer (25 mM Tris- HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1 mM DTT), and concentrated by ultrafiltration.

Generation of Anti-PTEN Antibody

Rabbit antisera was raised against purified Trx-mPTEN(151–403) protein (Antibodies, Inc., Davis, CA) and purified by preadsorption on Trx-coupled CNBr-Sepharose 4B followed by affinity purification on GST-PTEN fusion protein coupled to CNBr-Sepharose 4B. Low-affinity antibody was eluted in a pH 5.0 buffer, and high-affinity, PTEN-specific antibody (used in this paper) was eluted off the affinity column with 0.1 M glycine (pH 2.5).

Cell Lysis

For the generation of cytoplasmic cell lysates, cells were washed in ice-cold PBS and lysed by incubation on ice in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 50 mM NaF, 100 mM sodium vanadate, 1 mM benzamidine, and 5 μg/ml leupeptin. The lysates were centrifuged and supernatants analyzed.

For the generation of whole-cell lysates, the tissues were homogenized in a buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM MgCl₂, 1 mM EGTA, 0.5 mM CHAPS, 10% glycerol, 50 mM NaF, 100 mM sodium vanadate, 1 mM benzamidine, and 5 μg/ml leupeptin and

AGG-3' were separately cloned into the thioredoxin-hexahistidine tag (Trx) fusion vector pET32a (Novagen) and transfected into E. coli strain BL21(DE3)pLysS or BL21(DE3), respectively (Strategene, La jolla, CA).
incubated on ice for 30 min. The lysates were centrifuged and supernatants analyzed.

**Western Blot Analysis**

Proteins were resolved by 12.5% SDS-PAGE, electroblotted to PVDF membrane (Boehringer Manheim), blocked in 4% skim milk, 1× PBS, 0.05% Tween-20, and probed with primary antibodies. Anti-PTEN is described above; anti-phospho-S473 PKB/Akt and anti-PKB/Akt are from New England Biolabs. Following incubation with horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham), bound immunoglobulins were detected using enhanced chemiluminescence (Amersham).

**Analysis of Cell Death**

For analysis of cell death 3 × 10⁶ cells were plated and treated as indicated on the following day. Twenty-four hours after treatment, detached cells were collected, the remaining cells trypsinized, combined, and cell viability determined by negative 7-amino-actinomycin D staining (Schmid et al., 1994).

**Transient Transfection and Retroviral Infection**

Transient transfections of NIH 3T3 cells was performed using Lipofectamine (GIBCO-BRL) as per manufacturer's instructions. Briefly, 10⁶ cells were plated on 10 cm dishes and transfected the following day with a total of 10 μg of DNA mixed with the liposomes with the constructs indicated in figure legends. Retroviruses expressing HA-tagged PTEN were packaged within the ψNX-Eco cell line and mPTEN wildtype and mPTEN mutant cells infected as previously described (Hitoshi et al., 1998).

**Kinase and Phosphatase Assays**

PKB/Akt proteins were immunoprecipitated using an anti-PKB/Akt antibody (New England Biolabs) and analyzed for activity as described previously (Cross et al., 1995), except that the peptides were separated by tricine-SDS gel electrophoresis. Phosphorylated peptides were visualized by the Phosphorimager (Molecular Dynamix, Inc.) and incorporation of ³²P quantified using ImageQuant (MDI) software.

**Metabolic Cell Labeling and Lipid Extraction**

Incubation on ice for 30 min. The lysates were centrifuged and supernatants analyzed.

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**References**


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