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Tumor Suppression by PTEN Requires the Activation of the PKR-eIF2 α Phosphorylation Pathway

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Abstract

Inhibition of protein synthesis by phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF2) at Ser⁵¹ occurs as a result of the activation of a family of kinases in response to various forms of stress. Although some consequences of eIF2 α phosphorylation are cytoprotective, phosphorylation of eIF2 α by RNA-dependent protein kinase (PKR) is largely proapoptotic and tumor suppressing. Phosphatase and tensin homolog deleted from chromosome 10 (PTEN) is a tumor suppressor protein that is mutated or deleted in various human cancers, with functions that are mediated through phosphatase-dependent and -independent pathways. Here, we demonstrate that the eIF2 α phosphorylation pathway is downstream of PTEN. Inactivation of *PTEN* in human melanoma cells reduced eIF2 α phosphorylation, whereas reconstitution of *PTEN*-null human glioblastoma or prostate cancer cells with either wild-type PTEN or phosphatase-defective mutants of PTEN induced PKR activity and eIF2 α phosphorylation. The antiproliferative and proapoptotic effects of PTEN were compromised in mouse embryonic fibroblasts that lacked PKR or contained a phosphorylation-defective variant of eIF2 α . Induction of the pathway leading to phosphorylation of eIF2 α required an intact PDZ-binding motif in PTEN. These findings establish a link between tumor suppression by PTEN and inhibition of protein synthesis that is independent of PTEN's effects on phosphoinositide 3'-kinase signaling.

INTRODUCTION

Translational control of gene expression plays a critical role in the growth, proliferation, and differentiation of cells (1). There is a strong link between translational regulation and cancer because many of the genes that are essential for the growth and proliferation of cells are regulated at the translational level (2). Translation of messenger RNA (mRNA) is thought to be regulated mostly at the initiation step by modulation of various eukaryotic initiation factors (eIFs) including eIF4E and eIF2 (3). Exposure of cells to various extracellular stimuli, such as hormones, mitogens, and growth factors, leads to the activation of phosphoinositide 3-kinase (PI3K) and the recruitment of the serine-threonine kinase Akt [also known as protein kinase B (PKB)] to the plasma membrane, which results in the phosphorylation of Akt at Thr³⁰⁸ and Ser⁴⁷³ and its activation (4). Active Akt has a wide range of targets, one of the most important being the mammalian target of rapamycin (mTOR), a protein kinase that plays an essential role in the induction of cell growth and proliferation (5). Activation of mTOR results in the stimulation of protein synthesis by mediating, directly or indirectly, the phosphorylation of several proteins implicated in the translational control of gene expression (6). For example, phosphorylation of eIF4E-binding proteins (4E-BPs) contributes to stimulating the translation of mRNAs by weakening the interaction of 4E-BPs with the cap-binding protein eIF-4E, thus enhancing the initiation of cap-dependent translation (3). Phosphorylation of 4E-BPs has been documented in many types of human cancer and is considered a suitable target of chemotherapeutic intervention (7).

Metazoans respond to various forms of environmental stress by inducing the phosphorylation of the α subunit of eIF2, an essential component of the ternary complex that consists of eIF2, guanosine triphosphate (GTP), and methionyl initiator transfer RNA (Met-tRNA_i) (3). Each round of translation initiation requires the exchange of guanosine diphosphate (GDP) for GTP in the ternary complex, a process that is catalyzed by the guanine nucleotide exchange factor (GEF) eIF2B. Phosphorylation of eIF2 α at Ser⁵¹ converts eIF2 from a substrate to a competitive inhibitor of eIF2B and blocks the initiation of translation (3). In mammalian cells, eIF2 α is a common substrate for four kinases (8), which respond to different stress stimuli: the heme-regulated inhibitor kinase (HRI), which is activated by heme deficiency to regulate the translation of globin mRNA; the general control non-derepressible 2 kinase (GCN2), which is activated by amino acid deprivation; the RNA-dependent protein kinase (PKR)-related endoplasmic reticulum (ER) kinase (PERK), which responds to the accumulation of unfolded proteins in the ER and defines an important branch of the unfolded protein response (UPR); and PKR, which is an interferon (IFN)-inducible protein that is activated by binding to double-stranded RNA (dsRNA).

The gene encoding the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is mutated in various solid tumors, including gliomas, melanomas, prostate and breast carcinomas, and endometrial cancer (9). *PTEN* encodes a dual-specificity phosphatase that is capable of dephosphorylating lipids and peptides (9). Because the second messenger phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃] is its preferred substrate, PTEN functions by inactivating the PI3K pathway and inhibiting cell growth and proliferation (9). Some studies have shown that PTEN acts independently of PI3K signaling through mechanisms that involve posttranslational modifications of PTEN and its interactions with other proteins (10).

Here, we report that the PKR-eIF2 α phosphorylation pathway is induced by PTEN through mechanisms that are independent of its phosphatase activity and its capacity to block PI3K signaling. We showed that induction of the PKR-eIF2 α phosphorylation pathway required an intact PDZ-binding domain in the C-terminal region of PTEN. We further demonstrated

that activation of the PKR-eIF2 α phosphorylation pathway was essential for the antiproliferative and proapoptotic functions of PTEN.

RESULTS

Inactivation of PTEN in melanoma cells reduces the abundance of phosphorylated eIF2 α

In experiments with protein extracts from the human melanoma cell line UACC 903, which lacks *PTEN* (Fig. 1A), we observed a reduction in the abundance of eIF2 α phosphorylated at Ser⁵¹ (eIF2 α -pSer⁵¹) compared to that in normal melanocytes, which express *PTEN* (Fig. 1A). The cell lines 36A, 29A, and 37A are variants of the UACC 903 cell line and they contain only one chromosomal copy of wild-type *PTEN* (Fig. 1A) (11). We observed that reconstitution of these cell lines with *PTEN* increased the abundance of eIF2 α -pSer⁵¹ compared to that in the parental cells (Fig. 1A). Contrary to this, revertant cell lines from 36A cells, which lost the introduced chromosomal copy of *PTEN*, had a lower abundance of eIF2 α -pSer⁵¹ compared to that in cells that expressed one chromosomal copy of *PTEN* (Fig. 1A). These data established a tentative link between the expression of *PTEN* and induction of the phosphorylation of eIF2 α .

Conditional expression of wild-type *PTEN* induces the phosphorylation of eIF2 α

To better address the role of PTEN in the phosphorylation of eIF2 α , we used the *PTEN*-null human glioblastoma cell lines U87 and U251, which were engineered to express wild-type *PTEN* under the control of tetracycline-inducible promoter (12). The abundance of PTEN was substantially induced in doxycycline-treated cells compared to that in untreated cells, and this was accompanied by a decrease in the abundance of Akt phosphorylated at Ser⁴⁷³ (Akt-pSer⁴⁷³) and of phosphorylated 4E-BP1 (p4E-BP1) as a result of the inhibition of PI3K signaling (Fig. 1, B and C). The expression of wild-type *PTEN* coincided with an induction in the phosphorylation of eIF2 α at all time points during doxycycline treatment (Fig. 1, B and C). Treatment of parental U87 or U251 cells with doxycycline (fig. S1) did not affect the abundance of eIF2 α -pSer⁵¹, which showed the specificity of wild-type PTEN in inducing the phosphorylation of eIF2 α . The increased abundance of phosphorylated eIF2 α by wild-type PTEN became more evident when doxycycline-treated U87 or U251 cells were maintained in the absence of serum (Fig. 1, D and E), which indicated that mitogenic signaling by the presence of serum in culture media suppressed the phosphorylation of eIF2 α . Collectively, these data suggested that the expression of wild-type *PTEN* led to induction of the phosphorylation of eIF2 α .

Conditional expression of PTEN mutants induces the phosphorylation of eIF2 α

To determine whether the enzymatic activity of PTEN played a role in inducing the phosphorylation of eIF2 α , we used the doxycycline-inducible system to express the Gly¹²⁹→Glu (G129E) mutant of PTEN, which lacks lipid phosphatase activity, or the Cys¹²⁴→Ser (C124S) mutant of PTEN, which lacks both lipid and peptide phosphatase activity, in U87 cells. Doxycycline induced the production of the PTEN mutant proteins in either cell line, which coincided with an increase in the abundance of eIF2 α -pSer⁵¹ (Fig. 2, A and B). Doxycycline-induced expression of either PTEN C124S or PTEN G129E did not decrease the abundance of Akt-pSer⁴⁷³ (Fig. 2, A and B), nor did it reduce the extent of phosphorylation of ribosomal S6 and 4E-BP1 proteins (fig. S2, A and B), because both mutants were unable to impair PI3K-Akt signaling. Also, induction of the phosphorylation of eIF2 α by each of the PTEN mutants was stronger in the absence of serum (Fig. 2C), further indicating the inhibitory effects of mitogenic signaling on the phosphorylation of eIF2 α . Together, these data revealed that the enzymatic activities of PTEN were dispensable for inducing the phosphorylation of eIF2 α .

PTEN is an activator of the eIF2 α kinase PKR

To determine the kinase responsible for the phosphorylation of eIF2 α , we assessed the phosphorylation of PKR at Thr⁴⁴⁶, which is an autophosphorylation site with an essential role in the activation of the kinase (13). Conditional induction of either wild-type PTEN or PTEN mutants in U87 cells by doxycycline caused a strong increase in the phosphorylation of PKR at Thr⁴⁴⁶ either in the presence (Fig. 3A) or in the absence of serum (Fig. 3B). To verify the involvement of PKR, we targeted its expression in U87 cells by small inhibitory RNA (siRNA). We observed that knockdown of PKR prevented phosphorylation of eIF2 α in response to PTEN either in the presence (Fig. 3C) or in the absence of serum (Fig. 3D). Contrary to the results shown for PKR, the presence of wild-type PTEN did not lead to the phosphorylation of either GCN2 or PERK (fig. S3, A and B). Moreover, knockdown of PERK by siRNA did not prevent the phosphorylation of eIF2 α induced by wild-type PTEN (fig. S3C), which indicated that GCN2 and PERK were not involved in the induction of eIF2 α phosphorylation by PTEN. On the other hand, the ability of PTEN mutants to induce the activity of PKR suggested a PI3K-independent role of PTEN, which was further substantiated by the lack of phosphorylation of PKR at Thr⁴⁴⁶ in U87 cells treated with either LY294002, an inhibitor of PI3K, or rapamycin, an inhibitor of mTOR (Fig. 3E). Together, these data suggested that PKR mediated the induction of eIF2 α phosphorylation by PTEN.

The PTEN-mediated arrest of cell proliferation requires PKR

Next, we addressed the biological relevance of the activation of PKR and the phosphorylation of eIF2 α by PTEN. To do so, we performed colony formation assays in mouse embryonic fibroblasts (MEFs) from *PKR*^{+/+} and *PKR*^{-/-} mice in the absence or presence of ectopically expressed PTEN. Western blotting analyses of protein extracts from both cell types showed that the abundance of FLAG-tagged wild-type PTEN and FLAG-tagged PTEN C124S were similar, which was proportional to the amounts of plasmid DNA that were used to transfect the cells (Fig. 4). The presence of FLAG-PTEN led to the phosphorylation of PKR at Thr⁴⁴⁶ in the cytoplasm of transfected *PKR*^{+/+} MEFs, as detected by immunofluorescence (fig. S4). We observed that transfection with plasmids encoding wild-type PTEN or PTEN C124S substantially decreased the number of colonies formed in *PKR*^{+/+} MEFs compared to that of *PKR*^{-/-} MEFs (Fig. 4). Inhibition of colony formation in *PKR*^{+/+} MEFs was proportional to the amounts of plasmid DNA used to transfect the cells, indicating a dose-dependent response of PTEN in the inhibition of cell proliferation (Fig. 4A). Contrary to this, transfection of *PKR*^{-/-} MEFs with plasmids encoding wild-type PTEN or PTEN C124S did not affect the efficiency of colony formation compared to that in *PKR*^{-/-} MEFs transfected with vector DNA (Fig. 4B). Furthermore, the antiproliferative effects of either wild-type PTEN or PTEN C124S in *PTEN*-deficient human prostate cancer PC-3 cells were neutralized by the dominant-negative PKR mutant PKR Δ 6, which blocks autophosphorylation of PKR (14) (fig. S5). Collectively, these data suggested that PKR mediated the antiproliferative effects of PTEN in mouse and human cells.

Phosphorylation of eIF2 α is required for PTEN to mediate its antiproliferative effects and to induce apoptosis

To determine the role of the phosphorylation of eIF2 α in the function of PTEN, we expressed PTEN in MEFs from mice with a Ser⁵¹→Ala mutant knock-in of the gene encoding eIF2 α (eIF2 α A/A MEFs) and their isogenic wild-type counterparts (eIF2 α S/S MEFs) (15). We observed that transient expression of a plasmid encoding either wild-type PTEN or PTEN C124S decreased the viability of eIF2 α S/S MEFs compared to that of the same cells transfected with a plasmid encoding green fluorescent protein (GFP), which was used as control (Fig. 5A). The presence of either wild-type PTEN or PTEN C124S did not change the viability of eIF2 α A/A MEFs from that observed in the same cells transfected

with a plasmid encoding GFP (Fig. 5A), which indicated that phosphorylation of eIF2 α was required for the anti-proliferative effects of PTEN. Western blotting analyses of protein extracts showed a similar abundance of FLAG-tagged PTEN in eIF2 α A/A and eIF2 α S/S cells (Fig. 5B). We also noticed an increase in the extent of cleavage of caspase 3 (Fig. 5B) in eIF2 α S/S MEFs, indicating that apoptosis was more highly induced in eIF2 α S/S MEFs than in eIF2 α A/A MEFs. This finding was further supported by the decreased abundance of the antiapoptotic protein Bcl-xL by the PTEN proteins in eIF2 α S/S MEFs compared to that in eIF2 α A/A MEFs (Fig. 5B).

We further assessed apoptosis by more quantitative means in assays that measured the activity of caspase 3 with Ac-VEID-AFC as a substrate. We found that the activity of caspase 3 was induced by either wild-type PTEN or PTEN C124S in transfected eIF2 α S/S MEFs but not in transfected eIF2 α A/A MEFs (Fig. 5C). These findings were further substantiated in colony formation assays of eIF2 α S/S and eIF2 α A/A MEFs that were infected with retroviruses expressing PTEN proteins. Western blotting analyses of protein extracts from both cell types showed the equivalent abundance of wild-type PTEN and PTEN C124S (Fig. 5D). We found that both wild-type PTEN and PTEN C124S impaired the formation of colonies of eIF2 α S/S MEFs but not of eIF2 α A/A MEFs (Fig. 5D). By performing colony formation assays with *PTEN*-deficient PC-3 cells, we further documented the ability of wild-type PTEN and PTEN C124S to inhibit cell proliferation by inducing the phosphorylation of eIF2 α . We found that transfection of cells with a plasmid expressing the dominant-negative eIF2 α S51A mutant (16) rescued PC3 cells from the inhibitory effects of the PTEN proteins (fig. S6). Collectively, these findings documented an essential role for the phosphorylation of eIF2 α in the inhibition of cell proliferation and the induction of apoptosis by PTEN.

Induction of the PKR-eIF2 α phosphorylation pathway requires the PDZ motif of PTEN

The C terminus of PTEN is implicated in tumor suppression through its interactions with proteins that contain PDZ domains (10). Thus, we examined the role of the PTEN 401 mutant, from which the last three amino acid residues in the PDZ-binding motif of PTEN have been deleted (17), in the activation of PKR and the phosphorylation of eIF2 α . We found that PTEN 401 did not induce the phosphorylation of eIF2 α in U87 cells (Fig. 6A), nor did it lead to activation of PKR, as assessed by the detection of the phosphorylation of Thr⁴⁴⁶ (Fig. 6B and fig. S4); however, wild-type PTEN and PTEN C124S were able to do so efficiently. We further observed that PTEN 401 was unable to induce the activation of caspase 3 (Fig. 6C) in eIF2 α S/S or eIF2 α A/A MEFs, unlike wild-type PTEN and PTEN C124S, which did so in eIF2 α S/S MEFs only. These findings suggested that the PDZ-binding motif of PTEN was required for its antitumor effect through the induction of the PKR-eIF2 α phosphorylation pathway.

DISCUSSION

Our findings provide a link between the function of PTEN and the control of translation of mRNAs that leads to the inhibition of cell proliferation. It is well established that inhibition of PI3K signaling by PTEN inhibits protein synthesis by impairing the activity of mTOR and the phosphorylation of proteins involved in translational control (6). Here, we have demonstrated an alternative pathway used by PTEN to inhibit protein synthesis, which involves PKR and the phosphorylation of eIF2 α . In addition, our findings show that induction of the phosphorylation of eIF2 α is responsible for the inhibition of cell proliferation and the induction of apoptosis by PTEN independently of its phosphatase activity and its inhibition of the PI3K pathway.

In line with our findings, previous work provided strong evidence that the antitumor activities of PTEN are not always dependent on its catalytic activity (18). For example, the C2 domain of PTEN inhibits cell migration independently of its lipid phosphatase activity (19). Also, PTEN mutants unable to dephosphorylate lipids and proteins inhibit the invasion of human bladder cancer cells to the same degree as does wild-type PTEN (20). Moreover, PTEN plays an important role in the activation of p53 through physical interactions that enhance the DNA binding capacity of p53 in vitro and in vivo independently of its phosphatase activity (21). Furthermore, suppression of cellular transformation by the oncogenic micro-spherule protein 58 (MSP58), as well as maintenance of genomic stability, require PTEN, but are independent of its phosphatase activity (22, 23). That more than 40% of the naturally occurring mutations in PTEN that are found in cancer are localized to the C-terminal region indicates that PTEN has a broader role than simply antagonizing the PI3K pathway to fulfill its role as a tumor suppressor (9).

The ability of PTEN to induce the phosphorylation of PKR-eIF2 α required that it bound to proteins containing the PDZ domain; however, how the PDZ-binding property of PTEN mediates its antitumor activity is not well understood (10). PTEN interacts with the PDZ domain of membrane-associated guanylate kinase 2 (MAGI-2), a scaffold protein that improves the efficiency of PTEN-mediated signaling through the assembly of multi-protein complexes that stabilize PTEN and facilitate its ability to suppress the activation of Akt (24). Another study showed that the PDZ domain of the phosphatidylinositol 3,4,5-triphosphate RAC exchanger 2a (P-REX2a), a guanine nucleotide exchange factor (GEF) for the RAC guanosine triphosphatase, binds to PTEN and inhibits its lipid phosphatase activity, which results in the stimulation of PI3K signaling (25). We were unable to detect a direct physical interaction between PKR and PTEN by coimmunoprecipitation assays and confocal microscopic analysis (fig. S7). Thus, the ability of PTEN to activate PKR may require the recruitment of one or more PDZ domain-containing proteins that function as signaling intermediates leading to the activation of PKR (fig. S8). Although dsRNA is a physiological activator of PKR, the activation of PKR by proteins has been documented previously. For example, PKR-activating protein (PACT) is a dsRNA-binding protein that interacts with and activates PKR independently of dsRNA, leading to induction of the phosphorylation of eIF2 α and apoptosis (26).

There is a strong link between phosphorylation of eIF2 α and regulation of tumorigenesis. Early work demonstrated that dominant-negative mutants of PKR induce the malignant transformation of cultured cells (14, 27, 28), a phenotype that can be recapitulated by expressing the eIF2 α S51A mutant to prevent phosphorylation of eIF2 α (16). However, unlike in cultured cells, deletion of PKR (29, 30) or prevention of the phosphorylation of eIF2 α in mice (15) is not tumorigenic. This is likely due to a redundancy in the kinases that phosphorylate eIF2 α and to the complexity of the intracellular networks that converge on the phosphorylation of eIF2 α (8). It is now well established that kinases that phosphorylate eIF2 α are important determinants of cell survival and cell death. The prosurvival properties of these kinases are mediated, at least in part, by triggering the activation of PI3K (31) or nuclear factor κ B (NF- κ B) (32–34) or by inactivating p53 (35, 36) in response to distinct forms of stress. On the other hand, the proapoptotic effects of kinases are conveyed by prolonged phosphorylation of eIF2 α and severe inhibition of protein synthesis (31, 33). Among the kinases that phosphorylate eIF2 α , PKR plays a proapoptotic role (37). Thus, inactivation of the PKR-eIF2 α phosphorylation pathway may not be sufficient in itself to induce tumorigenesis, but when it is combined with the inactivation of a tumor suppressor pathway, such as PTEN, it may play an important role in cancer development.

MATERIALS AND METHODS

Plasmids

pCMV-FLAG-PTEN wild-type, pCMV-FLAG-PTEN C124S, and pCMV-FLAG-PTEN 401 constructs were described elsewhere (17), whereas pBabe retroviral constructs containing either wild-type PTEN or PTEN C124S complementary DNA (cDNA) were obtained from Addgene. The pRC-CMVempty vector and pRC-CMV-eIF2 α S51A were described elsewhere (16), as was pcDNA-PKR Δ 6 (14).

Cell culture and treatments

Tetracycline-inducible, PTEN-expressing U87 and U251 cells were described previously (12). The UACC 903 parental cell line, its variants (36A, 37A, and 29A), and revertant (36A-R1 and 36A-R2) extracts were described elsewhere (11). MEFs from isogenic *PKR*^{+/+} and *PKR*^{-/-} mice were described in previous studies (38). eIF2 α A/A and eIF2 α S/S MEFs were established as described previously (15).

Protein extractions, Western blotting, and immunoprecipitations

Protein extraction and Western blotting analyses were performed as described previously (31), as was immunoprecipitation of PKR (39). Rabbit polyclonal antibodies against Akt pS473, total Akt, S6-pSer235/236, and total S6 protein were obtained from Cell Signaling; rabbit polyclonal antibody against 4E-BP1 (40) and rabbit serum against pSer⁵¹ of eIF2 α were from Invitrogen; rabbit polyclonal antibody against eIF2 α was obtained from Santa Cruz; antibody against the FLAG epitope was from Sigma; mouse monoclonal antibody against PTEN was from BD; rabbit polyclonal antibody against PKR pT446 was from Cell Signaling; rabbit monoclonal antibody against PKR pT446 was from Abcam; mouse monoclonal antibody against PKR (F9) (41) and mouse monoclonal antibody against actin were from ICN; mouse monoclonal antibodies against PERK and GCN2 were generated in the laboratory; rabbit polyclonal antibody against GCN2 pT898; antibody against cleaved caspase 3, and antibody against Bcl-xL were obtained from Cell Signaling; antibody against Bax was from Santa Cruz. The secondary antibodies were horseradish peroxidase (HRP)-conjugated antibody against mouse immunoglobulin G (IgG) and HRP-conjugated antibody against rabbit IgG and were from Amersham Pharmacia Biotech. Quantification of the bands in the linear range of exposure was performed by densitometry with NIH Image 1.54 software.

Transient transfections and RNA interference

Transient transfection of 2×10^6 MEFs with 5 μ g of plasmid DNA encoding GFP, FLAG-PTEN wild type, or FLAG-PTEN C124S (17) was performed with the Amaxa Nucleofector system (MEF kit 1) according to the manufacturer's specifications. Transfection of 5×10^5 U87 cells with siRNA specific for human PKR (SMARTpool human PKR, Dharmacon), human PERK (SMARTpool human PERK, Dharmacon), or with scrambled siRNA (Dharmacon) was performed with the Amaxa Nucleofector system with kit T.

Colony formation assays and retroviral infections

MEFs (2×10^6 cells) were transfected in triplicate with pcDNA-GFP-zeocin^R and FLAG-PTEN wild type or FLAG-PTEN C124S plasmid DNAs at different ratios (1:0, 1:2, 1:5, and 1:10, respectively) with the Amaxa Nucleofector system as described earlier. After electroporation, cells were incubated at 37°C. After 24 hours, MEFs in culture were incubated with zeocin (400 μ g/ml) to select transfected cells. MEFs were also infected with retrovirus containing pBabe-puroL empty vector, PTEN wild-type, or PTEN C124S cDNA for three to five rounds of infection, followed by selection with puromycin (2.5 μ g/ml) until

colonies were formed. PC-3 cells were transfected with the TransIT-Prostate transfection kit (Mirus) according to the manufacturer's protocol. Transfected cells were selected with either zeocin (400 µg/ml) or G418 (500 µg/ml). For infection, retroviruses were collected from Φ Ap19 Vero packaging cells after they were transfected with 25 µg of DNA by the standard calcium phosphate method. For crystal violet staining, plates were washed twice with ice-cold 1× phosphate-buffered saline and then fixed with ice-cold methanol for 10 min. Cells were stained with 0.5% crystal violet solution (made in 25% methanol) at room temperature for 10 min followed by washing with deionized water. Plates were scanned and quantification of the clones was performed with Scion Image 4.0.3.2 software.

Caspase activity assay

Caspase 3 activity was assessed by fluorogenic assays with Ac-Val-Glu-Ile-Asp-7-Amino-4-trifluoromethylcoumarin (Ac-VEID-AFC; BIOMOL International) as the substrate. Activity was measured every 2 min for 1 hour at 37°C in Stennicke's buffer [20 mM Pipes (pH 7.2), 30 mM NaCl, 1 mM EDTA, 0.1% CHAPS, 10% sucrose, 10 mM dithiothreitol (DTT)] (42) and 10 µM Ac-VEID-AFC with a BioRad Fluoromark plate reader (excitation at 390 nm, emission at 538 nm) or a BMG Labtech FLUOstar Optima plate reader (excitation at 405 nm, emission at 520 nm). Fluorescence units were converted to the amount of moles of AFC released based on a standard curve of 0 to 50 µM free AFC. Cleavage rates were calculated from the linear phase of the assay.

Immunofluorescence studies

MEFs were transfected as described earlier and incubated with a 1:100 dilution of rabbit monoclonal antibody against PKR pThr⁴⁴⁶ (Abcam) and a 1:500 dilution of mouse monoclonal antibody against the FLAG epitope (Sigma); Alexa Fluor 546-conjugated antibody against rabbit IgG and Alexa Fluor 488-conjugated antibody against mouse IgG were used as fluorochromes. The nucleus was visualized after staining with 4,6-diamidino-2-phenylindole (DAPI, 0.05 µg/ml; Sigma) or with 0.5 µM DRAQ5 2λ (Biostatus). Images were captured on either a Zeiss microscope (100×) or a Zeiss LSM5 Pascal laser scanning confocal microscope (63× at room temperature). Zeiss AIM software (version 4) was used as acquisition software and ImageJ was used for image processing.

Statistical analysis

All quantitative variables are presented as means ± S.D. We compared the differences of three groups or more with one-way analysis of variance (ANOVA) (GraphPad Prism 5) and $P < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Increased phosphorylation of eIF2α as a result of PTEN expressed in human melanoma tumor cells and glioblastoma cells. (A) Protein extracts (50 μg) from normal melanocytes, melanoma tumor UACC 903 cells deficient in *PTEN*, variants of UACC 903 cells expressing a chromosomal copy of wild-type (WT) *PTEN* (36A, 29A, and 37A), and UACC 903 revertant (R) cells that lost the chromosomal copy of *PTEN* (R1 and R2) were analyzed by Western blotting with antibodies against eIF2α-pSer⁵¹, eIF2α, Akt-pSer⁴⁷³, PTEN, and actin. (B to E) U87 (B and D) and U251 cells (C and E) engineered to express WT *PTEN* under the control of a tetracycline-inducible promoter were treated with doxycycline (Dox, 1 μg/ml) for the indicated time points either in the presence (B and C) or in the absence (D and E) of serum. Protein extracts (50 μg) from untreated or doxycycline-treated cells were analyzed by Western blotting with antibodies against PTEN, Akt-pSer⁴⁷³, Akt, eIF2α-pSer⁵¹, eIF2α, and 4EBP1. The ratio of the amount of eIF2α-pSer⁵¹ relative to that of eIF2α protein for each lane is indicated. Data in (A) are representative of two experiments and those in (B) to (E) are representative of eight experiments.

Fig. 2.

Induction of the phosphorylation of eIF2α in the presence of phosphatase-defective mutants of PTEN. (A to C) U87 cells engineered to express the cDNA encoding PTEN C124S (A and C) or PTEN G129E (B and C) under the control of tetracycline-inducible promoter were treated with doxycycline (Dox, 1 μg/ml) for the indicated time points either in the presence (A and B) or in the absence (C) of serum. As a control, U87 cells containing the conditional inducible WT PTEN were included in (C). Protein extracts (50 μg) from untreated or doxycycline-treated cells were analyzed by Western blotting with antibodies against PTEN, Akt-pSer⁴⁷³, Akt, eIF2α-pSer⁵¹, and eIF2α. The ratio of the amount of eIF2α-pSer⁵¹ relative to that of eIF2α protein for each lane is indicated. Data shown are representative of eight experiments.

Fig. 3.

Activation of PKR by PTEN. U87 cells containing doxycycline-inducible WT PTEN (**A** and **B**), PTEN G129E (**A**), or PTEN C124S (**B**) were left untreated or were treated with doxycycline (Dox, 1 μ g/ml) for the indicated times. (**C** and **D**) U87 cells with conditionally inducible WT PTEN were subjected to treatment with siRNA specific for PKR or with scrambled (SCR) siRNA, which was used as control, in the presence (**C**) or absence of serum (**D**). (**E**) U87 cells with conditionally inducible WT PTEN were left untreated or were treated with either 20 μ M LY294002 (LY) or 20 nM rapamycin (Rapa) for the indicated times. The same cells were treated with doxycycline (Dox, 1 μ g/ml) as control. (**A**, **B**, and **E**) Protein extracts (500 μ g) were subjected to immunoprecipitation (IP) with an antibody against PKR (F9) followed by Western blotting analysis first with antibody against phospho-PKR Thr⁴⁴⁶ and second with antibody against PKR (F9). The ratio of the amount of phosphorylated PKR to that of total PKR protein for each lane is indicated in (**A**) and (**B**). (**C** to **E**) Protein extracts (50 μ g) were analyzed by Western blotting with antibodies against Akt-pSer⁴⁷³, eIF2 α -pSer⁵¹, eIF2 α , PKR (F9), phosphorylated S6 Ser^{235/236}, and PTEN. The ratio of the amount of eIF2 α -pSer⁵¹ to that of total eIF2 α protein for each lane is indicated. WCE, whole-cell extract. Data shown in (**A**), (**B**), (**C**), (**D**), and (**E**) are representative of three, three, four, four, and three experiments, respectively.

Fig. 4.

PKR is required for the inhibition of cell growth by PTEN. $PKR^{+/+}$ (A) and $PKR^{-/-}$ MEFs (B) were transfected with pcDNA-GFP-zeocin^R vector (Zeo control), as well as pcDNA vector bearing either FLAG-PTEN WT cDNA or FLAG-PTEN C124S cDNA in the presence of pcDNA-GFP-zeocin^R vector. The pcDNA vector encoding the PTEN inserts was used in 10-, 5-, or 2-fold excess over the control pcDNA vector bearing the zeocin resistance gene. Transfected MEFs were selected in zeocin (400 μ g/ml) for 3 weeks, and colonies were fixed in methanol and stained with crystal violet. Plates were scanned and quantification of the clones in the images was performed with Scion Image 4.0.3.2 software. Histograms show quantification of results from three independent experiments; error bars indicate the SD. The group difference was tested by ANOVA ($P < 0.0001$ for comparisons). Protein extracts (50 μ g) from cells harvested 48 hours after transfection were subjected to Western blotting analysis with antibodies against the FLAG epitope and actin. Data shown are representative of three experiments.

Fig. 5. Phosphorylation of eIF2 α mediates the antiproliferative and proapoptotic functions of PTEN. **(A)** eIF2 α S/S and eIF2 α A/A MEFs were transfected with pcDNA-GFP-zeocin^R vector (GFP), as well as pcDNA vector encoding either FLAG-PTEN WT cDNA or FLAG-PTEN C124S cDNA. Forty eight hours after transfection, cells were visualized by microscopy. **(B)** Protein extracts (50 μ g) from eIF2 α S/S and eIF2 α A/A MEFs treated as described in (A) were analyzed by Western blotting with antibodies against caspase-3, the FLAG epitope, Bcl-xL, Bax, or actin. **(C)** eIF2 α S/S and eIF2 α A/A MEFs were transfected as described for (A) and protein extracts (50 μ g) were analyzed for the activity of caspase 3 with Ac-VEID-AFC as substrate. The assay was performed in triplicate; error bars indicate SD, $n = 3$. The group difference was tested by ANOVA ($P < 0.0001$ for comparison). Protein extracts (50 μ g) were analyzed by Western blotting with antibodies against the FLAG epitope or actin. **(D)** eIF2 α S/S and eIF2 α A/A MEFs were infected with either empty pBABE retroviruses (as a control) or pBABE retroviruses containing either PTEN WT or PTEN C124S cDNAs. After selection in puromycin (2.5 μ g/ml) for 3 weeks, colonies were fixed and stained with crystal violet. Plates were scanned, and quantification of the clones on the images was performed with Scion Image 4.0.3.2 software. Histograms show quantification of results from three independent experiments; error bars indicate SD, $n = 3$. The group difference was tested by ANOVA ($P < 0.005$ for comparison). Protein extracts (50 μ g) were analyzed by Western blotting with antibodies against PTEN and actin. Data shown are representative of three experiments.

Fig. 6.

A PTEN mutant devoid of the PDZ motif is unable to activate the PKR-eIF2 α phosphorylation pathway. (**A** and **B**) U87 cells were transfected with either pcDNA vector alone or pcDNA vector containing PTEN WT, PTEN C124S, or PTEN 401 cDNAs. (**A**) Protein extracts (50 μ g) were analyzed by Western blotting with antibodies against PTEN, eIF2 α -pSer⁵¹, eIF2 α , or actin. The ratio of the amount of eIF2 α -pSer⁵¹ relative to that of total eIF2 α or actin for each lane is indicated. (**B**) Protein extracts (500 μ g) were used to immunoprecipitate PKR followed by Western blotting analysis of PKR phosphorylated at Thr⁴⁴⁶ or of total PKR protein. The ratio of the amount of phosphorylated PKR relative to that of total PKR protein for each lane is indicated. (**C**) eIF2 α S/S and eIF2 α A/A MEFs were transfected with pcDNA-GFP-zeocin^R vector (GFP), as well as pcDNA vector encoding either FLAG-PTEN WT cDNA, FLAG-PTEN C124S cDNA or FLAG-PTEN 401 cDNA. Forty eight hours after transfection, protein extracts (50 μ g) were analyzed for the activity of caspase 3 with Ac-VEID-AFC as a substrate. Histograms show quantification of results from three independent experiments; error bars indicate the SD, $n = 3$. The group difference was tested by ANOVA [$P < 0.0001$ for (C)]. Data shown are representative of three experiments.