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The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway

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ABSTRACT **The PTEN/MMAC1 phosphatase is a tumor suppressor gene implicated in a wide range of human cancers. Here we provide biochemical and functional evidence that PTEN/MMAC1 acts a negative regulator of the phosphoinositide 3-kinase (PI3-kinase)/Akt pathway. PTEN/MMAC1 impairs activation of endogenous Akt in cells and inhibits phosphorylation of 4E-BP1, a downstream target of the PI3-kinase/Akt pathway involved in protein translation, whereas a catalytically inactive, dominant negative PTEN/MMAC1 mutant enhances 4E-BP1 phosphorylation. In addition, PTEN/MMAC1 represses gene expression in a manner that is rescued by Akt but not PI3-kinase. Finally, higher levels of Akt activation are observed in human prostate cancer cell lines and xenografts lacking PTEN/MMAC1 expression when compared with PTEN/MMAC1-positive prostate tumors or normal prostate tissue. Because constitutive activation of either PI3-kinase or Akt is known to induce cellular transformation, an increase in the activation of this pathway caused by mutations in PTEN/MMAC1 provides a potential mechanism for its tumor suppressor function.**

PTEN/MMAC1 is a tumor suppressor gene implicated in multiple human tumors, including cancers of the prostate, breast, endometrium, glioblastoma, and melanoma (1–3). Loss of PTEN/MMAC1 function can occur through homozygous gene deletion, point mutation, or loss of expression (1, 2, 4). Several hereditary cancer-prone states such as Cowden disease and Bannayan-Zonana syndrome are associated with germline mutations in PTEN/MMAC1 (5, 6), and heterozygous disruption of PTEN/MMAC1 in mice leads to tumor formation in multiple tissues (7). Overexpression of PTEN/MMAC1 suppresses growth in a glioma cell line (8) and inhibits cell migration and spreading in fibroblasts, possibly through effects on focal adhesion kinase (9). Immunohistochemical studies show that PTEN/MMAC1 is present in the cytoplasm (4, 10), but its precise subcellular localization is not known. PTEN/MMAC1 contains a conserved catalytic motif found in multiple tyrosine phosphatases and has been shown to dephosphorylate a glutamine/tyrosine-rich peptide substrate as well as the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3, 4, 5)P_3] *in vitro* (11, 12). Because several other protein tyrosine phosphatases can antagonize or down-modulate specific signal transduction pathways (13–18), it is possible that PTEN/MMAC1 functions as a tumor suppressor by a similar mechanism, but the identity of the affected pathway(s) is unknown.

PtdIns (4)P and PtdIns (4, 5)P_2 are substrates of phosphoinositide 3-kinase (PI3-kinase), which generates PtdIns(3, 4)P_2 and PtdIns(3, 4, 5)P_3, respectively. PtdIns(3, 4)P_2 and PtdIns(3, 4, 5)P_3 participate with two other kinases, 3-phosphoinositide-dependent kinase 1 and 2, in the activation of the serine/threonine kinase Akt through binding its pleckstrin homology domain (19–26). The fact that PtdIns(3, 4, 5)P_3 is a substrate for PTEN/MMAC1 *in vitro* (12) and that PI3-kinase and Akt both play critical roles in a variety of growth factor signaling pathways (27–32) raises the possibility that PTEN/MMAC1 functions as a tumor suppressor through negative regulation of the PI3-kinase/Akt pathway. Here we show that increasing the level of PTEN/MMAC1 expression in cells reduces activation of endogenous Akt and phosphorylation of the downstream Akt target 4E-BP1. Conversely, inhibition of PTEN/MMAC1 function using a dominant negative allele enhances 4E-BP1 phosphorylation. One functional consequence of enhanced PTEN/MMAC1 expression is suppression of gene expression from prostate-specific antigen (PSA) and cytomegalovirus (CMV)-driven reporter constructs. PTEN/MMAC1-mediated repression of gene expression is rescued by activated Akt but not by activated PI3-kinase, suggesting that PTEN/MMAC1 is positioned in a signaling pathway between PI3-kinase and Akt. Human prostate cancer cell lines and xenografts lacking PTEN/MMAC1 expression have high levels of Akt activation compared with normal prostate tissue, whereas those prostate cancers that retain PTEN/MMAC1 expression do not. These results are consistent with the hypothesis that the transformation phenotype of loss of PTEN/MMAC1 function is a consequence of enhanced PI3-kinase/Akt pathway activation.

MATERIALS AND METHODS

cDNAs and Plasmids. The PTEN/MMAC1 cDNA was obtained by PCR using primers 5′-ACAGGCTCCCAGACATGACA-3′ and 5′-TCAGACTTTTGTAATTTGTGTATG-3′ and subcloned into the pCDNA3 expression vector (Invitrogen) and the retrovirus vector pSRαMSVtkNeo (33). The PTEN/MMAC1 C124S cDNA was created by PCR using primers 5′-AGCAATTCACTCTAAAGCTGGAA-3′ and 5′-TTCCAGCTTTAGAGTGAATTGCT-3′. Both constructs were confirmed by sequencing. The hemagglutinin (HA)-epitope tagged cDNA encoding 4E-BP1 was prepared by PCR using oligonucleotide primers as described (34), sequenced to confirm its identity and subcloned into pCDNA3. The CMV-luciferase plasmid was kindly provided by Lily Wu (University of California, Los Angeles). The PSA promoter/enhancer reporter construct (35), PEP expression construct (36), constitutively active PI3-kinase (37), dominant negative Akt (31),

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Abbreviations: PI3-kinase, phosphoinositide 3-kinase; PSA, prostate-specific antigen; PtdIns(3, 4, 5)P_3, phosphatidylinositol 3, 4, 5-trisphosphate; HA, hemagglutinin; GFP, green fluorescent protein; CMV, cytomegalovirus.

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and Myr-Akt (38) plasmids have been described. Transfections of LNCaP cells (39) were performed by using Tfx50 according to the manufacturer's instructions (Promega). 293T cells were transfected using calcium phosphate as described (40). PTEN/MMAC1 retrovirus was prepared by transient transfection of 293T cells as described (41). Rat-1 fibroblasts stably expressing wild-type PTEN/MMAC1 or PTEN/MMAC1 C124S were generated by infection with retrovirus and selection in G418 antibiotic.

RNA and Protein Analysis. RNA was prepared by using RNAzol and determined by Northern blot analysis. PTEN/MMAC1 expression was monitored by immunoblot using anti-PTEN antibody (4). Total endogenous Akt protein levels were measured by immunoblot using a pan-Akt antibody (New England Biolabs). Activated, phosphorylated Akt was measured using an antibody that specifically recognizes Ser-473 according to the manufacturer's instructions (New England Biolabs). Activated, phosphorylated Erk was measured by using a phospho-Erk antibody (Promega). Total endogenous Erk levels were measured by immunoblot using a pan-Erk antibody (Santa Cruz Biotechnology). Expression of transfected PI3-kinase was measured by immunoblot using an anti-Myc epitope antibody 9E10. Expression of transfected Akt was measured by immunoblot using anti-HA antibody. Phosphorylation of 4E-BP1 was monitored by altered electrophoretic mobility in immunoblot assays of whole-cell lysates using an anti-HA antibody. Immunoblot analysis for green fluorescent protein (GFP) expression was performed on whole-cell lysates using a commercially available antibody (CLONTECH) according to the manufacturer's instructions. GFP expression also was monitored by fluorescence microscopy and flow cytometry.

RESULTS AND DISCUSSION

To address the possibility that PTEN/MMAC1 functions as a tumor suppressor through negative regulation of the PI3-kinase/Akt pathway, we asked whether modulating the level of PTEN/MMAC1 protein affected biochemical activation of Akt. By using a phospho-specific antibody (anti-Akt Ser-473) that recognizes only activated Akt, we found a consistent 5-fold reduction in the level of phosphorylated endogenous Akt in 293T cells transfected with PTEN/MMAC1 without significant changes in the levels of total Akt protein (Fig. 1*A*). To determine the effect of PTEN/MMAC1 overexpression in stably expressing cells, Rat-1 fibroblasts were infected with retroviruses expressing wild-type PTEN/MMAC1 or a catalytically inactive mutant containing a cysteine to serine substitution at position 124 (PTEN/MMAC1 C124S), a change known to impair phosphatase activity (11). After selection for 2 weeks in G418 antibiotic, populations of fibroblasts expressing wild-type PTEN/MMAC1, PTEN/MMAC1 C124S, or Neo alone were obtained (Fig. 1*B*). Cells were starved of serum for 24 hr then challenged with serum for 30 min to activate endogenous Akt. Serum-induced Akt activation was severely blunted in wild-type PTEN/MMAC1-expressing Rat-1 cells but not in the Neo control cells or the PTEN/MMAC1 C124S-expressing cells (Fig. 1*C*). These results indicate that expression of PTEN/MMAC1 represses basal levels of Akt activation and blocks growth-factor-induced Akt activation in a phosphatase-dependent fashion.

To determine whether PTEN/MMAC1 expression affected other signaling pathways, we examined the activation of Erk kinases in the Rat-1 populations expressing wild-type PTEN/MMAC1 or PTEN/MMAC1 C124S. Erk is activated by a signaling cascade involving the Raf and Mek kinases and is not known to be influenced by phosphoinositides (42). By using an antibody that specifically recognizes activated, phosphorylated Erk, we observed no significant differences in basal Erk activation in Rat-1 cells expressing wild-type or PTEN/MMAC1 C124S (Fig. 1*B*). This result is consistent with previous *in vitro* studies suggesting that the Erk kinases are not substrates for the PTEN/MMAC1 phosphatase (11) and suggests that the effects of PTEN/MMAC1 on Akt activation are specific.

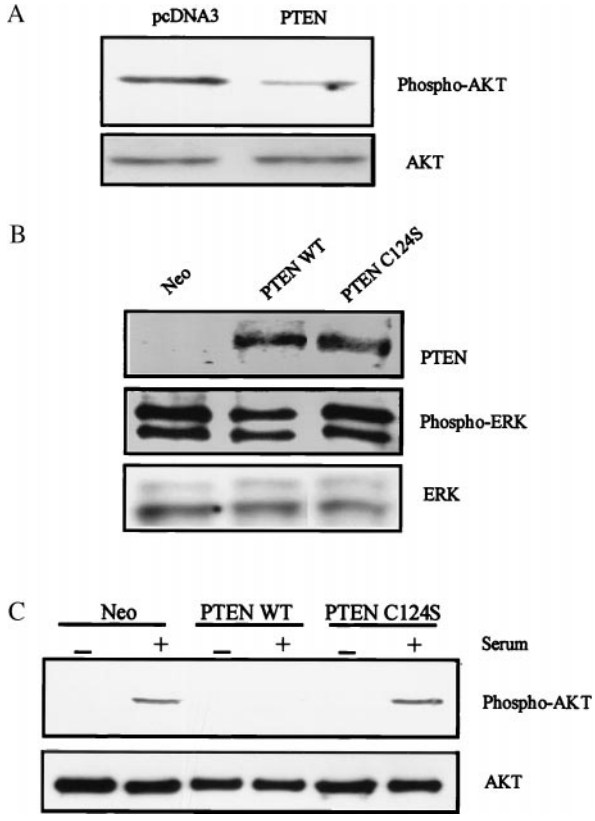

FIG. 1. PTEN/MMAC1 impairs growth factor-induced activation of Akt. (*A*) 293T cells were transfected with vector or wild-type PTEN/MMAC1. Endogenous levels of phosphorylated (*Top*) and total (*Bottom*) Akt protein levels were measured by immunoblot using an antibody that specifically recognizes Ser-473 (*Top*) and a pan-Akt antibody (*Bottom*). (*B*) Populations of Rat-1 cells stably expressing a Neo control vector, wild-type PTEN/MMAC1 or PTEN/MMAC1 C124S were established by retroviral infection and drug selection in G418. The expression of PTEN/MMAC1 was detected using an antibody against endogenous PTEN/MMAC (*Top*).The basal levels of activated Erk were analyzed by using an antibody that specifically recognizes phosphorylated, activated Erk (*Middle*). Total Erk protein was measured by using a pan-Erk antibody (*Bottom*). (*C*) Whole-cell lysates were prepared from Rat-1 cells stably expressing neo vector, wild-type PTEN, or PTEN C124S that were starved of serum for 24 hr and after re-exposure to serum for 30 min. Phosphorylated (*Top*) and total (*Bottom*) Akt levels were measured by immunoblot as in *A*.

To determine whether the effects of PTEN/MMAC1 on Akt phosphorylation also led to down-regulation of an Akt-dependent signal, we examined the phosphorylation status of 4E-BP1, a known biochemical target of the PI3-kinase/Akt pathway (34). 4E-BP1 is a translation repressor that inhibits ribosome assembly on the 5′-cap structure of mRNAs by binding the eIF4E initiation factor (43, 44). When phosphorylated, 4E-BP1 is no longer capable of binding to eIF4E and translation of 5′-capped mRNAs can proceed efficiently. As expected, transfection of a constitutively activated allele of PI3-kinase or Akt enhanced the level of 4E-BP1 phosphorylation, which is easily detected because of its slower electrophoretic mobility. Wild-type PTEN/MMAC1 blocked the PI3-kinase-induced band shift (Fig. 2*A*), whereas the catalytically inactive mutant had no effect (Fig. 2*B*). In contrast, PTEN/MMAC1 did not impair Akt-induced 4E-BP1 phosphorylation, suggesting that PTEN/MMAC1 functions bio-

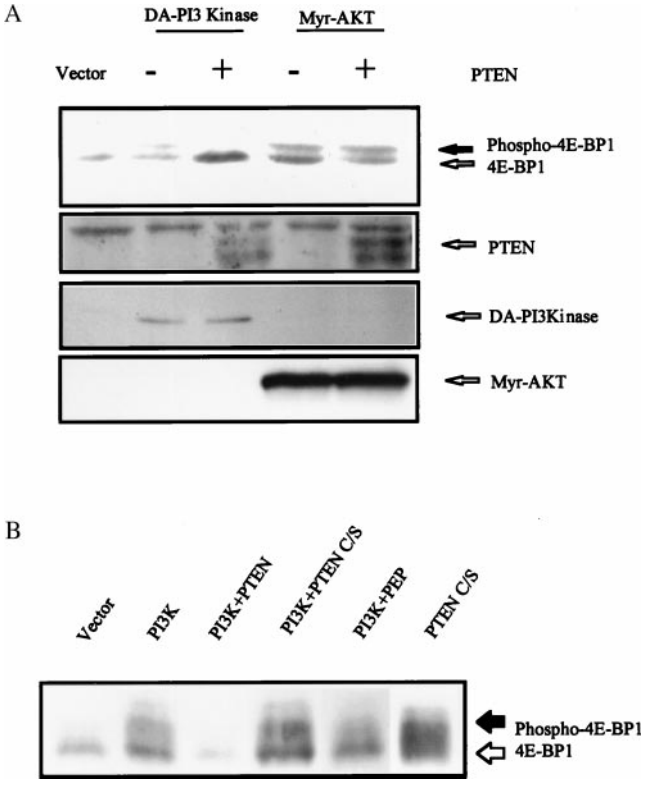

FIG. 2. PTEN/MMAC1 blocks phosphorylation of the translation repressor 4E-BP1, a downstream target of the PI3-kinase/Akt pathway. (*A*) 293T cells were transfected with 4E-BP1 in the presence or absence of wild-type PTEN/MMAC1 and either Myr-Akt or constitutively active PI3-kinase then starved of serum for 48 hr. Phosphorylation of 4E-BP1 was monitored by altered electrophoretic mobility in immunoblot assays of whole-cell lysates using an anti-HA antibody. Expression of PTEN/MMAC1, PI3-kinase, and Myr-Akt was monitored by immunoblot using anti-PTEN antibody, anti-Myc antibody, and anti-HA antibody respectively. (*B*) 293T cells were transfected with 4E-BP1 in conjunction with plasmids noted at the top of each lane. Phosphorylation of 4E-BP-1 was monitored as described in *B*.

chemically between PI3-kinase and Akt. The effects of PTEN/MMAC1 in this assay cannot be explained by nonspecific effects of phosphatase overexpression because another phosphatase PEP [proline-, glutamic acid-, serine-, and threonine-rich (PEST)-domain phosphatase] (36) had no effect on 4E-BP1 phosphorylation (Fig. 2*B*).

Having established that overexpression of PTEN/MMAC1 impairs biochemical activation of the Akt pathway, we asked whether inhibiting endogenous PTEN/MMAC1 function would enhance Akt pathway activation. The catalytically inactive PTEN/MMAC1 C124S mutant, which functions as a dominant inhibitor of wild-type PTEN/MMAC1 (12) (data not shown), activated 4E-BP1 phosphorylation in the absence of PI3-kinase (Fig. 2*B*). Based on the combination of gain-of-function studies through overexpression and loss-of-function studies using a dominant negative allele, we conclude that PTEN/MMAC1 functions biochemically to down-regulate the PI3-kinase/Akt pathway.

Because unphosphorylated 4E-BP1 inhibits protein translation, we asked whether overexpression of PTEN/MMAC1 led to any changes in gene expression. Because of our interest in the role of PTEN/MMAC1 in prostate cancer (4), we performed these studies in LNCaP cells, a prostate cancer line that lacks PTEN/MMAC1 expression because of gene mutation (1, 2). First we asked whether PTEN/MMAC1 affected signaling through the androgen receptor. Remarkably, wild-type PTEN/MMAC1 consistently repressed both basal and androgen-inducible levels of the androgen-responsive PSA promoter/enhancer, whereas the catalytically inactive PTEN/MMAC1 C124S mutant had no effect (Fig. 3*A*). In the course of these experiments we also noted reduced expression of a control plasmid used to measure transfection efficiency (CMV-enhanced GFP) in PTEN/MMAC1-transfected cells (Fig. 3*B*). Similar reductions in gene expression were obtained by using CMV-luciferase as the reporter (Fig. 3*A*) as well as in U87 glioma cells (data not shown), indicating that this effect is not specific to LNCaP cells or prostate cancer.

Because PTEN/MMAC1 can suppress growth in U87 glioma cells (8), one potential explanation for our results is that PTEN-induced cellular toxicity leads to a general decrease in gene expression. However, in a series of kinetic experiments we observed 16- to 50-fold suppression of CMV-luciferase within 6–18 hr after transfection (Fig. 3*C*). To determine whether these effects were a consequence of cell death or growth arrest, LNCaP cells were cotransfected with CMV-GFP and either control vector, wild-type PTEN/MMAC, or PTEN/MMAC1 C124S and analyzed by flow cytometry after staining with propidium iodide and/or by fluorescence mi-

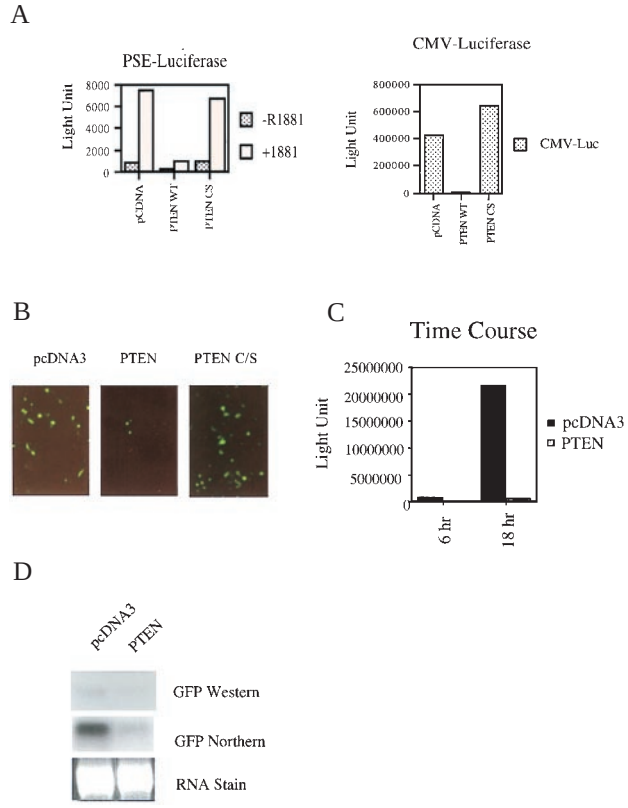

FIG. 3. PTEN/MMAC1 suppresses gene expression from the PSA and CMV promoters. (*A*) LNCaP prostate cancer cells were transfected with PSA enhancer (PSE)-luciferase or CMV-luciferase in conjunction with vector, wild-type PTEN/MMAC1 or PTEN/MMAC1 C124S. Luciferase activity in PSE luciferase-transfected cells was analyzed 48 hr after stimulation with 1 nM concentration of the synthetic androgen R1881 (DuPont) vs. control. CMV-luciferase-transfected cells were not stimulated. (*B*) LNCaP cells were transfected with CMV-enhanced GFP and vector, wild-type PTEN/MMAC1, or PTEN/MMAC1 C124S and examined by fluorescence microscopy after 48 hr. Flow cytometry analysis confirmed successful transfection of all plates with reduced brightness in the wild-type PTEN/MMAC1-transfected cells (data not shown). (*C*) LNCaP cells were transfected with CMV-luciferase and analyzed at 6 and 18 hr after transfection. (*D*) LNCaP cells transfected with CMV-GFP and vector or wild-type PTEN/MMAC1 were analyzed after 48 hr for GFP expression by immunoblot (*Top*) or Northern blot analysis (*Middle*). 28S RNA levels were measured by ethidium bromide staining. Immunoblot analysis for GFP protein expression was performed on whole-cell lysates using a commercially available antibody (CLONTECH).

croscopy at various time intervals from 6 to 48 hr after transfection. In three independent experiments, no differences in cell cycle profile or sub-G1 DNA content were observed at any timepoint after gating on the green cells, nor was there any morphologic evidence of increased apoptosis or reduced viability in the wild-type PTEN/MMAC1-transfected cells. The success of the transfections was documented by expression of GFP in ≈30% of the cells and by immunoblot analysis confirming PTEN/MMAC1 protein expression (data not shown). These results indicate that the effects on gene expression are unlikely to be a consequence of PTEN/MMAC1-induced toxicity.

To determine whether PTEN/MMAC1-mediated suppression of gene expression is the result of effects on transcription or translation or both, we compared RNA and protein levels of GFP in cells cotransfected with CMV-GFP and a control vector or wild-type PTEN/MMAC1. Wild-type PTEN/MMAC1 caused a reduction in the level of GFP mRNA and GFP protein (Fig. 3*D*), indicating that the effects of PTEN/MMAC1 on gene expression are complex and may not be mediated entirely through inhibition of translation. One possibility is that a PTEN/MMAC1-mediated block to translation impairs the synthesis of critical transcription factors, leading to a secondary reduction in mRNA levels. Alternatively, transcription from the PSA and CMV promoters used in these studies may require the activity of a specific signaling pathway that is the target of PTEN/MMAC1 repression.

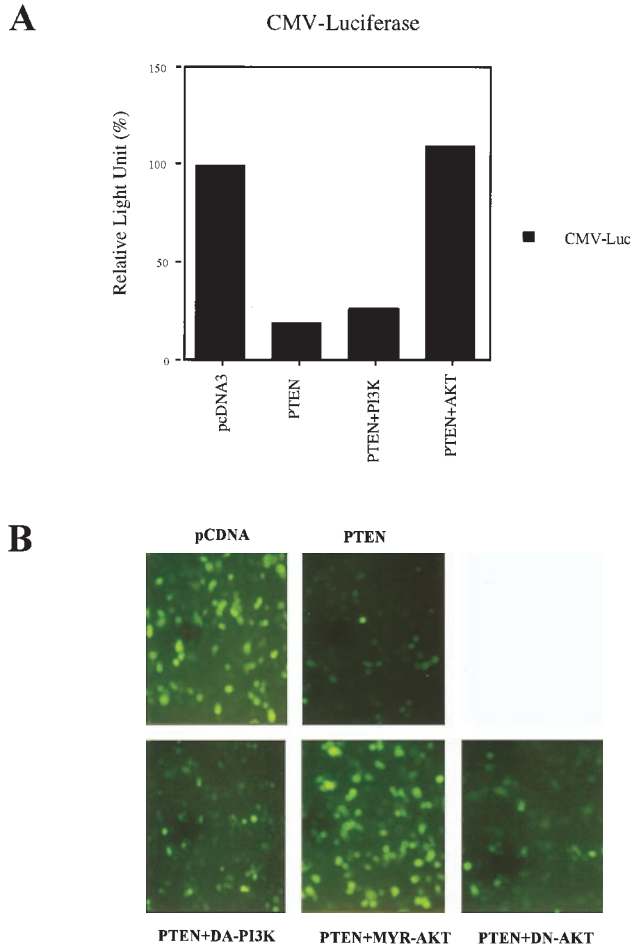

FIG. 4. Akt but not PI3-kinase rescues PTEN/MMAC1-mediated suppression of gene expression from the CMV promoter. (*A*) Transfections were performed as described in Fig. 3*A* using CMV-luciferase as the reporter. (*B*) Transfections were performed as described in Fig. 3*B* using CMV-enhanced GFP and visualized by fluorescence microscopy.

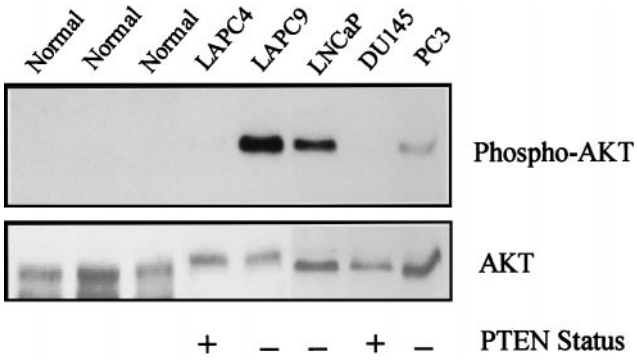

FIG. 5. Enhanced levels of Akt activation in human prostate cancers lacking PTEN expression. Whole-cell lysates were prepared from the human prostate cancer cell lines LNCaP, DU145, PC3, and prostate cancer xenografts LAPC-4 and LAPC-9, which were passaged as subcutaneous tumors in male severe combined immunodeficient mice, and from normal human prostate tissue from three individual patients. The levels of phosphorylated (*Top*) and total (*Bottom*) Akt were measured by using antibodies as described in Fig. 1. The status of the PTEN gene in each tumor is designated + (wild type) or – (mutant) as determined (1, 2, 4).

To address these general issues and the specific possibility that the suppressive effect of PTEN/MMAC1 on gene expression resulted from inhibition of the PI3-kinase/Akt pathway, we asked whether activated Akt could rescue cells from the PTEN/MMAC1-induced block to gene expression. Analogous to the results from biochemical studies of 4E-BP1 phosphorylation, Myr-Akt restored gene expression from CMV-luciferase and CMV-enhanced GFP plasmids in PTEN/MMAC1-transfected cells, whereas activated PI3-kinase did not (Fig. 4*A*). Similarly, expression of CMV-GFP was restored by Myr-Akt when cells were examined by fluorescence microscopy (Fig. 4*B*). The kinase activity of Akt is required for rescue from PTEN/MMAC1-block to gene expression because a kinase-inactive mutant, Akt K179M, was unable to restore gene expression (Fig. 4*B*). Further study is required to determine whether the ability of Akt to rescue cells from PTEN/MMAC1-mediated repression of gene expression is primarily a consequence of effects at the transcriptional or translational level. Because PI3-kinase and Akt can affect gene expression through modulation of transcription as well as translation (45–49), it is probable that both processes play a role. In either case, our results provide functional evidence that the PTEN/MMAC1-mediated block to gene expression occurs downstream of PI3-kinase but proximal to Akt, precisely the point at which the putative PTEN/MMAC1 substrate PtdIns(3,4,5)P_3 is believed to function.

One prediction based on our observations that PTEN/MMAC1 inhibits the PI3-kinase/Akt pathway is that tumors lacking PTEN/MMAC1 function as a consequence of gene mutation may have elevated levels of Akt activation. We tested this hypothesis by using a set of human prostate cancer cell lines and xenografts grown as tumors in severe combined immunodeficient mice whose PTEN/MMAC1 status has been previously characterized (1, 2, 4). The level of activated endogenous Akt was significantly higher in LNCaP, PC3, and LAPC-9 tumors, three prostate cancer lines that lack PTEN/MMAC1 protein expression caused by gene mutation, when compared with DU145 and LAPC-4 tumors, which express wild-type PTEN/MMAC1 protein, or to normal prostate tissue (Fig. 5). All samples contained comparable levels of total Akt protein. These results establish a correlation between the loss of PTEN/MMAC1 expression and activation of Akt and indicate that the Akt pathway is up-regulated in human cancers containing PTEN/MMAC1 mutations.

Because loss of PTEN/MMAC1 function is a common molecular abnormality in human cancer, defining its mecha-

nism of growth suppression is of critical importance. The functional and biochemical evidence presented here from expression studies and from analysis of human prostate tumors demonstrate that PTEN/MMAC1 functions to inhibit the PI3-kinase/Akt pathway, presumably through dephosphorylation of the PI3-kinase substrate PtdIns(3,4,5)P_3. Because constitutive activation of either PI3-kinase or Akt is known to induce cellular transformation (50–53), loss of function mutations in the negative regulators of this pathway are likely to produce a similar phenotype. Therapeutic strategies that target the PI3-kinase/Akt pathway may be particularly effective in cancers lacking PTEN function because these tumors show higher levels of pathway activation.

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