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PTEN Induces G_1 Cell Cycle Arrest and Decreases Cyclin D3 Levels in Endometrial Carcinoma Cells[1]

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ABSTRACT

Inactivating mutations in the *PTEN* tumor suppressor gene occur in approximately 30–50% of endometrial carcinomas. PTEN is a phosphatase that negatively regulates the phosphoinositide 3-kinase signaling pathway, including the downstream effector AKT. To evaluate the role of PTEN in endometrial growth regulation, we expressed wild-type or mutant PTEN in endometrial carcinoma cell lines. As expected, expression of exogenous PTEN decreased levels of activated AKT in all cell lines examined. However, PTEN induced a G_1 cell cycle arrest specifically in endometrial carcinoma cells that lack endogenous wild-type PTEN. Growth of cells containing wild-type PTEN was unaffected by exogenous PTEN expression. Growth arrest required a functional phosphatase domain but not the PDZ interaction motif of PTEN. Overall levels of CIP/KIP and INK4 family members, the known inhibitory regulators of the G_1 phase of the cell cycle, were unchanged. However, PTEN induced a specific reduction of cyclin D3 levels and an associated increase in the amount of the inhibitor $p27^{KIP1}$ complexed with CDK2. Enforced expression of cyclin D3 abrogated the PTEN-induced cell cycle arrest. Although PTEN signaling directly regulates $p27^{KIP1}$ levels in some settings, in endometrial carcinoma cells, PTEN expression indirectly regulated $p27^{KIP1}$ activity by modulating levels of cyclin D3. These data support multiple mechanisms of PTEN-induced cell cycle arrest.

INTRODUCTION

The *PTEN* tumor suppressor gene is inactivated by mutations in tumors of the endometrium, brain, prostate, and others (1–7). PTEN is a lipid phosphatase with the ability to dephosphorylate the 3′ position of the signaling molecule PIP3[3] (8). Consistent with this finding, structural analysis of PTEN revealed a unique phosphate binding pocket that accommodates PIP3 (9). Although there may also be protein substrates for PTEN, including FAK and SHC (10, 11), functional analysis of mutant PTEN suggests that the lipid phosphatase, but not protein phosphatase activity, is essential for tumor suppression (12). Through direct regulation of PIP3 levels, PTEN negatively regulates the PI3K signaling pathway, which transduces extracellular growth regulatory signals to intracellular mediators of growth and cell survival (reviewed in Ref. 13). Accordingly, in tumors, inactivating mutations in PTEN led to increased activity of AKT/PKB, one of the most well-characterized downstream effectors of PI3K (14, 15). The COOH-terminal three amino acids of PTEN, threonine, lysine, and valine (TKV), comprise a consensus for binding to proteins containing a PDZ domain, a modular protein-protein interaction motif. Although PTEN interacts specifically with the PDZ domains in MAGI 2 and MAGI 3 (16, 17), it is unclear if such interactions are general regulators of PTEN activity in all cell types. Homologues of PTEN in *Caenorhabditis elegans* and Drosophila also function to attenuate the PI3K pathway, but the PDZ interaction motif is not conserved (18–21).

The ability of PTEN to counteract PIP3 signals is consistent across different cell backgrounds, although the consequences of this activity vary widely. Introduction of PTEN into tumor cell lines results in apoptosis (22–25), anoikis (26, 27), or growth arrest (28–34). In some cases, cell cycle arrest was only revealed when cells were grown in low serum (30). *In vivo*, PTEN activity is particularly important in growth regulation of endometrium (35). PTEN mutations occur at the earliest stages of endometrial carcinogenesis (3, 36–38), whereas such mutations are late events in the tumorigenic progression in other tissues such as brain (4, 39, 40) and prostate (41, 42). Familial syndromes with germ-line mutations in *PTEN* have a predisposition to develop endometrial hyperplasia and carcinoma, as well as breast and thyroid cancers, macrocephaly, and hamartomas in multiple tissues with widely variable penetrance (43). Finally, 100% of female mice heterozygous for an inactivating mutation in Pten display complex atypical endometrial hyperplasia with pathological features very similar to the human precancerous lesions (44, 45).

In this report, we analyzed the consequence of expressing exogenous wild-type or mutated PTEN in endometrial carcinoma cell lines. PTEN induced cell cycle arrest in endometrial carcinoma cells lacking endogenous PTEN but not in a cell line containing wild-type PTEN. Growth arrest required a functional phosphatase domain, but not the PDZ interaction domain of PTEN, and was mediated through altered levels of cyclin D3.

MATERIALS AND METHODS

Expression Constructs. The full-length coding sequence of wild-type PTEN was amplified by reverse transcription-PCR using cDNA template from human cerebellum. PCR was used to introduce replacement of cysteine 124 with alanine (C124A) and deletion of the three COOH-terminal amino acids (ΔTKV). The correct wild-type and mutant sequences were confirmed by automated sequence analysis. Wild-type and mutant PTEN cDNAs were cloned into the retroviral expression vector MSCV-IRES-GFP described previously (46). This MSCV-based vector drives expression of a bicistronic message containing the PTEN insert followed by an IRES and GFP. All control experiments using empty vector lack the PTEN insert but still express GFP. Cyclin D3 cDNA (47) was excised from Bluescript as an *Eco*RI fragment and cloned into the *Eco*RI site of the retroviral expression vector MSCV-IRES-YFP (a gift from Elio Vanin, St. Jude Children's Research Hospital) to allow expression of a bicistronic message containing cyclin D3, an IRES, and YFP. The helper plasmid pPAM3-E, kindly provided by Elio Vanin, drives expression of the *gag* and *pol* retroviral genes, and an additional helper plasmid, pSRa-G (48), expresses the VSV envelope protein (VSV-G).

Cell Lines. Endometrial carcinoma cell lines AN3CA, RL-95, and Hec1A were obtained from American Type Culture Collection (Manassas, VA). Sequence analysis of PTEN in these cell lines was performed as described previously (3). 293T cells (49) were used to produce high titer retrovirus. AN3CA, RL-95, and 293T cells were cultured in DMEM (Life Technologies, Inc., Bethesda, MD), and Hec1A cells were cultured in McCoy's 5A medium (Life Technologies, Inc., Bethesda, MD). All media were supplemented with

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[3] The abbreviations used are: PIP3, phosphatidylinositol-3,4,5-trisphosphate; PI3K, phosphoinositide 3-kinase; GFP, green fluorescent protein; VSV, vesicular stomatitis virus; FACS, fluorescence-activated cell sorting; CDK, cyclin-dependent kinase; YFP, yellow fluorescent protein; MSCV, murine stem cell virus; IRES, internal ribosome entry site; MOI, multiplicities of infection.

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10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Production of High Titer VSV-G Pseudotyped Retrovirus and Retroviral Transduction. 293T cells were plated at a density of 3×10^6 cells per 100-mm plate and transfected the following day. For each retrovirus, the transfection mix containing 4.5 μg of pMSCV expression plasmid, 4.5 μg of pPAM3-E helper plasmid, 3 μg of pSRa-G helper plasmid, and 32 μl of FuGene (Roche Molecular Biochemicals) was added to 10 ml of medium in a 100-mm plate. Twenty-four h after transfection, the medium was changed, and conditioned medium containing replication-incompetent retrovirus was harvested at 48 and 72 h after transfection and filtered through a 0.45 μm filter. The retroviral titers generated with this procedure, as determined by FACS analysis of transduced NIH-3T3 cells, were approximately 2×10^6 particles/ml. For retroviral transduction, cells were incubated 24 h with undiluted conditioned medium containing retrovirus and 6 μg/ml Polybrene (Sigma Chemical Co., St. Louis, MO). To analyze the effects of retrovirus at different MOI, the conditioned medium was diluted with culture medium. Polybrene was added to a final concentration of 6 μg/ml. Efficiency of retroviral transduction was assessed by FACS analysis for GFP expression.

Western Blotting and Immunoprecipitation. For Western blot analysis, 1×10^6 cells were seeded onto 60-mm plates and transduced with 1 ml of retrovirus. Forty-eight h after retroviral transduction, cells were washed with PBS and lysed in RIPA buffer containing protease inhibitor mixture (Boehringer Mannheim), 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 1 mM Na_3VO_4, and 50 mM β-glycerophosphate. Protein concentration was quantitated using a modified Bradford assay (Bio-Rad protein assay), and 50 μg of total protein were resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose filters. For Western blot detection, filters were incubated for 1 h with blocking solution (5% milk in TBST) and then with primary antibodies: 2 μg/ml anti-PTEN (AB-2; Oncogene Research Products); 1:1000 anti-phospho-AKT (Ser-473; New England Biolabs); 1:1000 anti-AKT (New England Biolabs); 1:1000 anti-$p27^{KIP1}$ (Transduction Labs); 1:200 anti-$p57^{KIP2}$ (Santa Cruz Biotechnology); 1:200 anti-$p21^{CIP1}$ (Santa Cruz Biotechnology); 1:250 anti-β-actin (Sigma Chemical Co.); 1:200 anti-CDK2 (Santa Cruz Biotechnology); 1:250 anti-cyclin D1 (Santa Cruz Biotechnology); 1:250 anti-cyclin D2 (Santa Cruz Biotechnology); or 1:250 anti-cyclin D3 (Santa Cruz Biotechnology) according to manufacturer's instructions. Proteins were detected by incubation with horseradish peroxidase-conjugated antirabbit or antimouse (1:5000; Amersham Life Sciences), followed by enhanced chemiluminescence (ECL from Amersham Life Sciences; or SuperSignal chemiluminescent substrate from Pierce). For immunoprecipitation of CDK2 complexes, 1×10^6 cells were seeded onto 100-mm plates and transduced with 10 ml of retrovirus-containing medium. Forty-eight h after transduction, cells were lysed and immunoprecipitated using anti-CDK2 (Santa Cruz Biotechnology) as described previously (50). Five μg of antibody were used to immunoprecipitate CDK2 complexes from cell extracts containing 300 μg of protein. Immunoprecipitated complexes were resolved by SDS-PAGE and detected by Western blot analysis as indicated above.

Cell Growth and Survival. For cell growth analysis, cells were seeded at a density of 2.5×10^4 cells/well in 24-well plates. Cells were transduced with 1 ml of retrovirus/well, and the cell number was determined on the indicated days after transduction by crystal violet staining as described previously (51). All experiments were performed in triplicate. Transduction efficiency was determined by FACS analysis for GFP expression using cells from three wells 72 h after transduction. For cell cycle analysis, 1×10^6 cells were transduced with retrovirus and analyzed 48 h after retroviral transduction. GFP-positive cells were isolated by FACS and stained with propidium iodide. DNA fluorescence was determined by FACS, and the percentages of cells within the G_1, S, and G_2-M phases of the cell cycle were determined as described previously (52). For cell cycle analysis of cotransduced cells, 1×10^6 cells in a 60-mm plate were transduced with 1.5 ml each of PTEN and cyclin D3 retroviruses or the appropriate empty vectors containing GFP or YFP. Twenty-four h after retroviral transduction, GFP and YFP double-positive cells were isolated by FACS, and cell cycle analysis was determined as above. *In situ* detection of cells undergoing apoptosis was performed by *in situ* end labeling staining as described by the manufacturer (Oncogene Research Products) or by FACS analysis of FITC-conjugated annexin V staining (Boehringer Mannheim).

RESULTS

The PDZ Interaction Domain Is Dispensable for the PTEN-mediated Decrease in Activated AKT. Three endometrial adenocarcinoma cell lines were selected to analyze the mechanism of PTEN-mediated tumor suppression in endometrial cancer. Two of the cell lines contained mutations in *PTEN* such that no wild-type protein was made. AN3CA contained a deletion of a single bp in codon 130, leading to truncation of the protein at codon 133 with a loss of the second *PTEN* allele, and RL-95 contained two mutations, a 1-bp deletion in codon 321 and a 1-bp insertion in codon 323. The Hec1A cell line contained wild-type *PTEN* and served as a control in which loss of PTEN did not contribute to tumorigenesis. Wild-type or mutated PTEN, or empty vector, was introduced into cells using VSV-G pseudotyped retroviruses. A phosphatase-inactive form of PTEN was engineered by substitution of cysteine 124, a conserved residue in the phosphatase motif that is required for catalytic activity, with alanine (C124A). A three-amino acid deletion at the COOH terminus of PTEN (ΔTKV) was introduced to remove the consensus PDZ-interaction motif. Unlike larger COOH-terminal deletions (53, 54), removal of the three COOH-terminal amino acids does not appear to change protein stability, because this mutated protein was expressed at approximately equal levels compared with wild-type PTEN (Fig. 1, *Lanes 2* and *4*). The retroviral expression vectors are constructed such that a single bicistronic message is transcribed, encoding PTEN and GFP, which are independently translated. Thus, visualization of GFP indicates *PTEN* coexpression. FACS analysis for GFP verified that >95% of cells were transduced by retrovirus (data not shown).

Wild-type and mutated PTEN were efficiently expressed in all three cell lines after retroviral transduction (Fig. 1, *top panel*). Levels of wild-type PTEN expressed in AN3CA and RL-95 cells were somewhat higher than the endogenous level expressed in Hec1A cells (Fig. 1, *Lanes 2* and *6* compared with *9*). PTEN dephosphorylates PIP3 and antagonizes the PI3K signaling cascade and activity of AKT, a major downstream effector of PI3K. AKT activity is modulated by phosphorylation of threonine 308 or serine 473. Consistent with this characterized pathway, AN3CA and RL-95 cells, which lack wild-

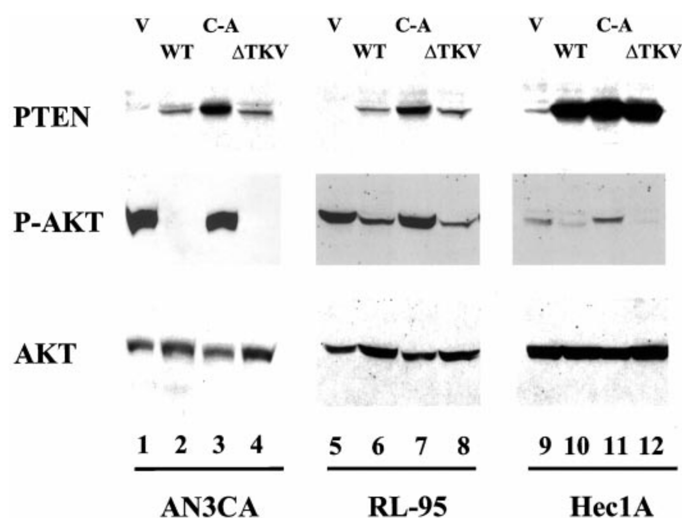

Fig. 1. PTEN induces a decrease in AKT phosphorylation. Endometrial carcinoma cell lines AN3CA (*Lanes 1–4*), RL-95 (*Lanes 5–9*), and Hec1A (*Lanes 9–12*) were transduced with retrovirus containing vector only (*V*; *Lanes 1, 5,* and *9*), wild-type PTEN (*WT*; *Lanes 2, 6,* and *10*), mutant PTEN (C124A) lacking phosphatase activity (*C-A*; *Lanes 3, 7,* and *11*), or a COOH-terminally deleted PTEN (ΔTKV; *Lanes 4, 8,* and *12*). Cell extracts were resolved by SDS/PAGE and immunoblotted with anti-PTEN (*top panel*), anti-phospho-AKT (*middle panel*), or anti-AKT (*lower panel*).

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type PTEN, contained elevated levels of phospho-AKT compared with Hec1A cells, which retain wild-type PTEN. Endogenous phospho-AKT levels in cells transduced with empty vector (Fig. 1, *middle panel, Lanes 1, 5,* and *9*) are equivalent to untransduced cells (data not shown). Reintroduction of wild-type or PTEN ΔTKV effectively decreased levels of phospho-AKT, whereas expression of PTEN C124A, which lacks phosphatase activity, did not alter levels of phospho-AKT (Fig. 1, *middle panel*). These effects were observed in all three cell lines and were therefore independent of the endogenous PTEN status. The total level of AKT within the cell was not substantially affected by exogenous PTEN expression (Fig. 1, *lower panel*).

PTEN Suppresses Growth of Endometrial Carcinoma Cells Lacking Endogenous PTEN. The PI3K pathway is a central mediator of growth control signals and can regulate survival and/or growth rate depending on the context of the signal and cell type. Accordingly, reintroduction of PTEN into tumor cell lines has been reported to interfere with growth through a number of different mechanisms including apoptosis, anoikis, and cell cycle arrest (22, 25–30, 32–34, 55, 56). To evaluate the effect of wild-type or mutant PTEN on the growth rate of the three endometrial carcinoma cell lines, we transduced cells with retrovirus expressing wild-type or mutant PTEN, or the vector expressing GFP alone, and determined the cell number daily for 6 days after transduction. As for the previous experiment, FACS analysis showed that >95% of cells were transduced. Wild-type PTEN markedly inhibited growth of AN3CA and RL-95 cells (Fig. 2, *A* and *B, circles*), and deletion of the PDZ-interaction motif did not affect the ability of PTEN to suppress growth (Fig. 2, *A* and *B, triangles*). However, phosphatase activity was required for growth suppression, because cells transduced with PTEN C124A grew at a similar rate to cells transduced with vector alone or untransduced cells (Fig. 2, *A* and *B, diamonds* and *squares*). In Hec1A cells, which contain endogenous PTEN, introduction of wild-type or PTEN ΔTKV showed a minimal effect on growth (Fig. 2*C*), despite the ability to decrease levels of phospho-AKT (Fig. 1). Therefore, PTEN specifically arrested growth of cells that lacked endogenous wild-type PTEN.

These data suggest that the phosphatase activity of PTEN is critical for its growth-suppressive effects, whereas PDZ-domain interactions are dispensable. Recently, PTEN has been shown to interact with the PDZ-domain containing proteins MAGI 2 and MAGI 3, which localize to epithelial cell tight junctions. A functional enhancement of PTEN activity in the presence of these interacting proteins was only apparent at extremely low levels of PTEN expression (transfections of 1–30 ng of PTEN expression plasmid; Refs. 16 and 17). The levels of wild-type or ΔTKV protein expressed by retroviral transduction in AN3CA and RL-95 cells were slightly higher than the endogenous level of PTEN in Hec1A cells (Fig. 1, *top panel, Lanes 2, 4, 6,* and *8* compared with *9*). To determine whether deletion of the PDZ-interaction motif could inhibit growth suppression at lower levels of PTEN expression, we decreased the MOI by titrating the amount of PTEN retrovirus used over a 32-fold range. Although undiluted virus transduced 92% of the cells, 80% of cells were transduced by a 2-fold dilution of virus, 52% of cells were transduced at a 4-fold dilution of virus, 25% of cells were transduced at an 8-fold dilution of virus, and 7% of cells were transduced with a 32-fold dilution of virus (Fig. 3). Using these conditions, we determined the number of cells 72 h after transduction, a point at which growth suppression was easily detected in previous experiments (Fig. 2). Growth suppression by PTEN ΔTKV was as effective as wild-type PTEN at all dilutions in which a PTEN-mediated effect could be observed in AN3CA and RL-95 cells (Fig. 3 and data not shown), including conditions in which only 25% of cells were transduced. Therefore, the PDZ interaction domain did not play a role in growth suppression of these endometrial carcinoma

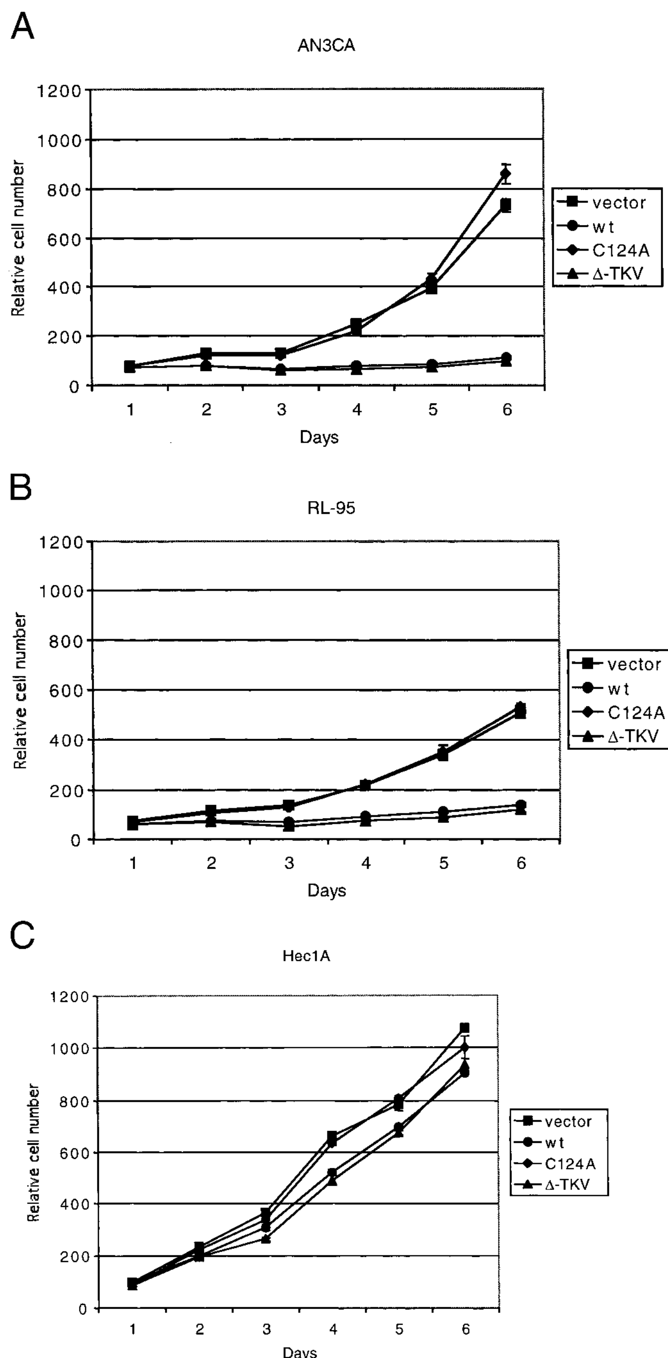

Fig. 2. PTEN suppresses growth of endometrial cell lines lacking endogenous PTEN. Endometrial carcinoma cell lines AN3CA (*A*), RL-95 (*B*), and Hec1A (*C*) were transduced with retrovirus containing empty vector (*squares*), wild-type PTEN (*circles*), C124A mutant PTEN (*diamonds*), or ΔTKV PTEN (*triangles*). Cell number was quantified daily for 6 days after transduction using crystal violet staining and is shown as the relative percentage of cells compared with untransduced cells on day 1. Average results from three independent experiments are shown; *bars,* SD.

cells. As expected, growth of Hec1A cells was unaffected by PTEN expression at all MOIs tested (data not shown).

PTEN Suppresses Growth through a G_1 Cell Cycle Arrest. The lack of growth in PTEN-transduced cells may result from cell cycle effects, cell death, or a combination of the two processes. The cell cycle distribution of cells transduced with wild-type or mutant PTEN, or a vector control, was determined by FACS analysis of propidium iodide stained cells, a measure of DNA content. Wild-type PTEN induced an increase in the number of cells in the G_0-G_1 phase of the

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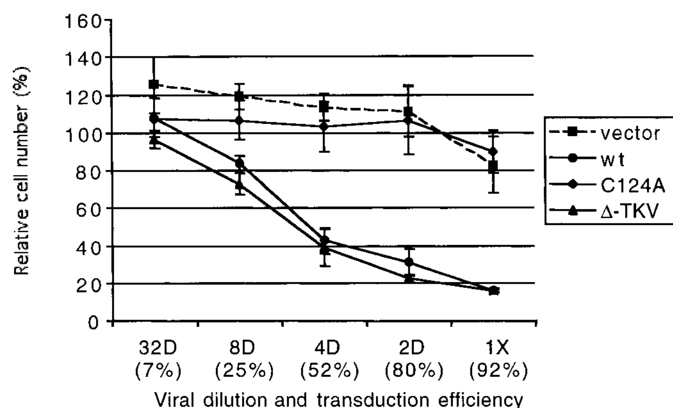

Fig. 3. Deletion of the PDZ interaction domain does not affect PTEN-mediated growth inhibition. AN3CA cells were transduced with retrovirus containing empty vector (*squares*), wild-type PTEN (*circles*), C124A mutant PTEN (*diamonds*), or ΔTKV PTEN (*triangles*) over a 32-fold range of retroviral titers. Transduction efficiency was determined by FACS analysis for GFP. The fold dilution of virus and percentage of cells transduced is indicated along the *X axis*. The cell number was quantified 72 h after transduction using crystal violet staining and is shown as the percentage relative to untransduced cells at the same time point. Average results from three independent experiments are shown; *bars,* SD. *wt,* wild type.

cell cycle with a concomitant decrease in the proportion of cells in the S and G_2-M phases in AN3CA and RL-95 cells (Fig. 4). Deletion of the PDZ interaction domain (ΔTKV) resulted in a similar cell cycle arrest, whereas abrogation of phosphatase activity (C124A) did not affect the cell cycle. As expected from the growth rates shown in Fig. 2, introduction of wild-type or mutant PTEN did not have an appreciable effect on the cell cycle distribution in Hec1A cells (Fig. 4).

To determine the contribution of cell death to PTEN-induced growth arrest, we used two different methods. *In situ* end labeling, a variation of the TUNEL assay, detects DNA fragmentation associated with apoptosis. As an alternative measure, immunostaining for Annexin V, a marker of apoptotic death, was determined by FACS. No appreciable change in the number of apoptotic cells in populations transduced with wild-type or mutant PTEN compared with vector controls was found using either method (data not shown). Thus, PTEN-induced growth suppression does not involve apoptosis but is attributable to cell cycle arrest in endometrial carcinoma cells deficient in PTEN.

PTEN Induces Complex Formation between p27^{KIP1} and CDK2. We examined the expression of inhibitory regulators of the cell cycle to determine the mechanism of PTEN-induced cell cycle arrest. Introduction of wild-type or mutant PTEN did not affect expression of the CDK inhibitors p27^{KIP1} or p57^{KIP2} as determined by Western blot analysis (Fig. 5 and data not shown). Expression of p21$^{WAF1/CIP1}$ was low or undetectable in all three cell lines and remained unchanged after transduction with wild-type or mutant PTEN (data not shown). The INK4 family of inhibitors negatively regulate the cyclin D-dependent kinases, CDK4 and CDK6. None of these family members, p16^{INK4A}, p15^{INK4B}, p18^{INK4C}, or p19^{INK4D}, showed changes in protein expression after introduction of wild-type or mutant PTEN (data not shown).

The activity of CDKs is regulated through differential associations with activators or inhibitors (57). CIP/KIP proteins are potent inhibitors of cyclin-CDK2 complexes. To determine whether the proportion of CDK2 that was complexed with inhibitory proteins changed in response to PTEN expression, we determined the amount of the CDK inhibitors p27^{KIP1} or p57^{KIP2} that coimmunoprecipitated with CDK2 from cells transduced with a vector control or wild-type or mutant PTEN. Although equivalent quantities of CDK2 were immunoprecipitated (Fig. 6, *lower panel*), introduction of wild-type or ΔTKV PTEN induced a substantial increase in the amount of p27^{KIP1} that was associated with CDK2 (Fig. 6, *Lanes 2, 4, 6,* and *8*). This redistribution of p27^{KIP1} only occurred in AN3CA and RL-95 cells in which cell cycle arrest was observed and was not seen in cells

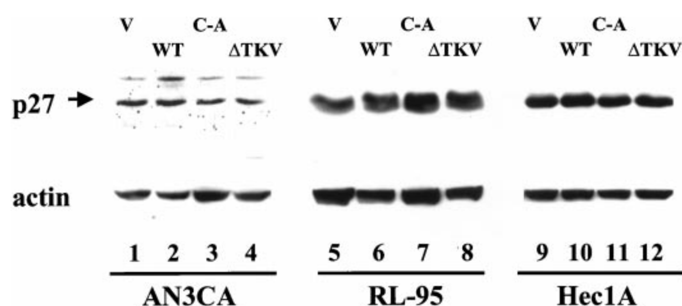

Fig. 5. PTEN expression does not affect levels of p27^{KIP1}. Cells were transduced as described in Fig. 1. Cell extracts were resolved by SDS-PAGE and immunoblotted with anti-p27^{KIP1} (*upper panel*) or anti-β-actin (*lower panel*).

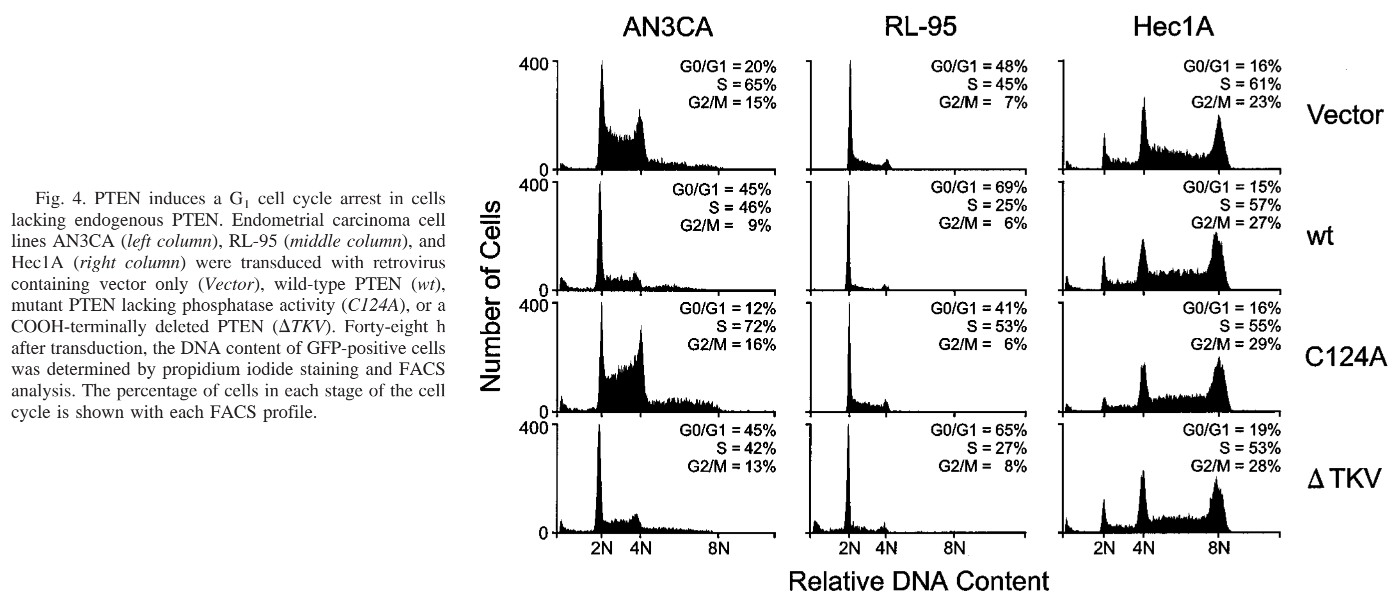

Fig. 4. PTEN induces a G_1 cell cycle arrest in cells lacking endogenous PTEN. Endometrial carcinoma cell lines AN3CA (*left column*), RL-95 (*middle column*), and Hec1A (*right column*) were transduced with retrovirus containing vector only (*Vector*), wild-type PTEN (*wt*), mutant PTEN lacking phosphatase activity (*C124A*), or a COOH-terminally deleted PTEN (Δ*TKV*). Forty-eight h after transduction, the DNA content of GFP-positive cells was determined by propidium iodide staining and FACS analysis. The percentage of cells in each stage of the cell cycle is shown with each FACS profile.

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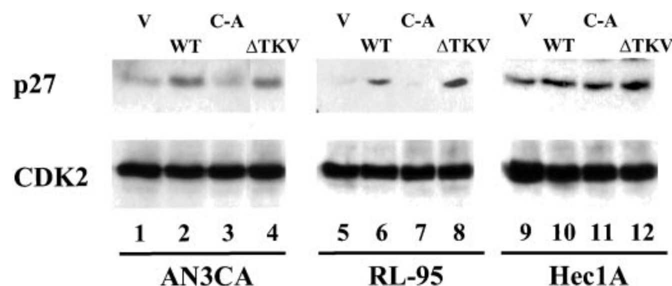

Fig. 6. PTEN expression induces complex formation between $p27^{KIP1}$ and CDK2. Cells were transduced as described in Fig. 1. Protein complexes immunoprecipitated from cell extracts with anti-CDK2 were resolved by SDS-PAGE and immunoblotted with anti-$p27^{KIP1}$ (*upper panel*) or anti-CDK2 (*lower panel*).

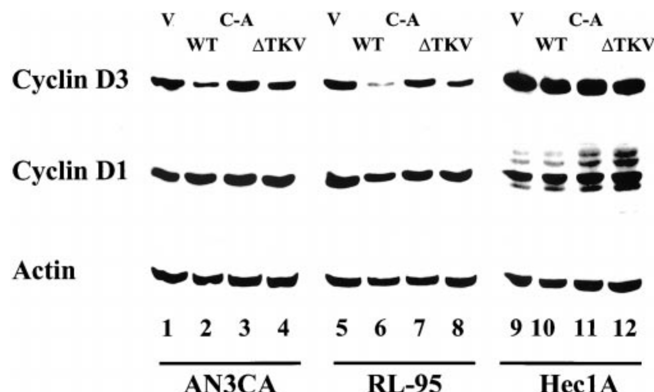

Fig. 7. PTEN expression decreases cyclin D3 levels. Cells were transduced as described in Fig. 1. Cell extracts were resolved by SDS-PAGE and immunoblotted with anti-cyclin D3 (*upper panel*), anti-cyclin D1 (*middle panel*), or anti-β-actin (*lower panel*).

transduced with PTEN C124A, which lacks phosphatase activity, or in Hec1A cells. Expression of the introduced PTEN proteins was verified by Western blot (data not shown). $p57^{KIP2}$ could not be detected in CDK2 immunoprecipitates, and $p21^{WAF1/CIP1}$ was not expressed at detectable levels in these cells (data not shown). Therefore, the altered association between CDK2 and $p27^{KIP1}$ is likely to be the major effector of PTEN-induced cell cycle arrest in these cells.

$p27^{KIP1}$serves a dual purpose in cell cycle regulation; it facilitates activation of cyclin D-CKD4/6 complexes and inhibits activity of cyclin-CDK2 complexes (57). The redistribution of $p27^{KIP1}$ into complexes with CDK2 is generally driven by levels of $p27^{KIP1}$ or by changes in the components of the cyclin D-CDK4/6 complex. As the expression levels of all of the known G_1 cell cycle inhibitors remained unchanged in the presence of exogenous PTEN, we analyzed expression of the cyclin D family members (D1, D2, and D3; Fig. 7). Expression of cyclin D1 remained relatively constant, regardless of PTEN expression. Cyclin D2 levels were low to undetectable in AN3CA and RL-95 cells and detectable but unchanged by PTEN expression in Hec1A cells (data not shown). However, cyclin D3 was decreased in AN3CA and RL-95 cells transduced with wild-type or ΔTKV PTEN but was not changed by expression of the phosphatase-inactive C124A. In Hec1A cells, which were not growth arrested by any version of PTEN, cyclin D3 levels remained unchanged (Fig. 7). Therefore, decreased levels of cyclin D3 were directly associated with cell cycle arrest.

If decreased cyclin D3 expression is required for PTEN-induced cell cycle arrest, then enforced expression of cyclin D3 should overcome the cell cycle block. We tested this hypothesis in AN3CA cells in which the cell cycle arrest was most pronounced. In addition to the PTEN-GFP bicistronic retrovirus used previously, we generated retroviruses expressing a single bicistronic message encoding cyclin D3 and YFP, which were independently translated. AN3CA cells were cotransduced with the GFP and YFP retroviruses expressing PTEN and cyclin D3, respectively, or with the corresponding empty vector controls. The cell cycle distribution of cells coexpressing GFP and YFP was determined by FACS. As shown previously, expression of wild-type PTEN induced a dramatic increase in the number of cells in the G_0-G_1 phase of the cell cycle compared with cells transduced with vector alone. Expression of cyclin D3 increased the proportion of cells in S-phase with a concomitant decrease in the number of cells in G_0-G_1, consistent with its role in cell cycle progression. Similarly, coexpression of PTEN and cyclin D3 resulted in an increase in the proportion of cells in S-phase compared with cells transduced with vector controls (Fig. 8). Thus, coexpression of cyclin D3 was sufficient to overcome PTEN-induced cell cycle arrest.

DISCUSSION

PTEN expression induced cell cycle arrest in endometrial carcinoma cells lacking endogenous PTEN, whereas the growth of a cell line containing wild-type PTEN was unaffected. In AN3CA and RL-95 cells, inhibition of growth paralleled decreases in the levels of phospho-AKT. In contrast, growth of Hec1A cells was unaffected by PTEN expression despite decreased levels of phospho-AKT. However, the levels of phospho-AKT were already very low in these cells. It is likely that Hec1A cells have sustained an alternative mutation(s) downstream of AKT that contributed to tumorigenesis in the presence of persistent wild-type PTEN. In agreement with this possibility, the downstream effects of PTEN expression, decreased cyclin D3 levels and a concomitant increase in association of p27KIP1 with CDK2, were not observed in Hec1A cells. Because all tumors and tumor cell lines contain multiple mutations, it is possible that not all endometrial tumors that contain wild-type PTEN would have the same resistance to PTEN induced cell cycle arrest observed in Hec1A cells. Although we did not observe apoptosis, a previous study found that adenovirus-mediated delivery of PTEN induced apoptosis in endometrial carcinoma cell lines (23). These differences may reflect the relative levels of PTEN introduced by retroviral compared with adenoviral transduction.

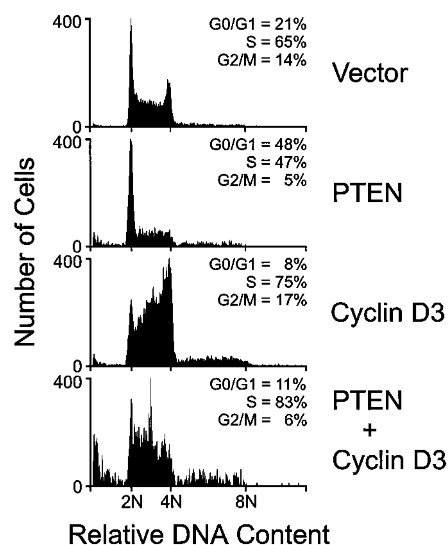

Fig. 8. Enforced expression of cyclin D3 abrogates the PTEN-induced G_1 arrest. Cells were transduced with retrovirus expressing GFP and YFP vectors (*Vector*), PTEN and YFP vector (*PTEN*), GFP vector and cyclin D3 (*Cyclin D3*), or PTEN and cyclin D3 (*PTEN + Cyclin D3*). Twenty-four h after transduction, the DNA content of cells coexpressing GFP and YFP was determined by propidium iodide staining and FACS analysis. The percentage of cells in each stage of the cell cycle is shown with each FACS profile.

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The PDZ interacting motif was dispensable for PTEN-mediated growth suppression in our system, whereas the phosphatase activity was essential. Interactions with the PDZ domain-containing proteins MAGI 2 and MAGI 3 only revealed a functional enhancement of PTEN activity at extremely low levels of expression (16, 17). However, we were unable to detect differences in the growth suppression by wild-type and PTEN ΔTKV, even at the lowest levels of PTEN expression at which this activity was observed. Under these conditions, we transduced 25% of the cell population. The vast majority of those cells would receive only a single retroviral particle/cell. The studies with MAGI proteins measured PTEN activity by evaluating changes in phosphorylation of a cotransfected AKT (16, 17), whereas we examined the functional growth-suppressive effects. As shown by the Hec1A cells, a decrease in the level of phospho-AKT does not necessarily correlate with growth suppression. However, the lack of a substantial PDZ-mediated effect in our system may reflect an absence of expression of functionally relevant PDZ proteins in endometrium or at least in the carcinoma cell lines that we were studying. The PDZ interaction domain may also mediate other activities of PTEN, such as platelet-derived growth factor-induced membrane ruffling (58). In any event, the PDZ interaction motif of PTEN was not required for the cell cycle arrest found in this system.

Altered regulation of the CDK inhibitor $p27^{KIP1}$ has been identified as a major target of PTEN and AKT signaling in several different experimental systems. However, the mode of regulation varies depending on the cell type and the experimental context. For example, absence of Pten was associated with decreased levels of $p27^{Kip1}$ in Pten−/− embryonic stem cells but not Pten−/− mouse embryo fibroblasts (59). In human glioblastoma cells and in thyroid carcinoma cells, expression of wild-type PTEN induced an increase in $p27^{KIP1}$ (29, 32, 60) and an associated cell cycle arrest. Similarly, we found that expression of PTEN in endometrial carcinoma cells can induce a G_1 arrest by changing the proportion of CDK2 complexed with the inhibitor $p27^{KIP1}$. However, the mechanism underlying this change is not attributable to changes in $p27^{KIP1}$ expression. Activated AKT can negatively regulate AFX/Forkhead-mediated transcription of *KIP1* RNA (61), and PTEN expression leads to activation of Forkhead transcription factors and induction of p27KIP1 in renal carcinoma cells (62). Therefore, one would predict that $p27^{KIP1}$ would be down-regulated in cells containing mutant PTEN and corresponding increased levels of phospho-AKT. This is not the case in the endometrial cell lines examined in our study. RL-95 cells, which lack PTEN, contain much higher levels of $p27^{KIP1}$ than Hec1A cells containing wild-type PTEN. Therefore, there is not a direct association between PTEN status and $p27^{KIP1}$ levels.

The D-type cyclins are regulated by mitogenic cues (63), and inhibition of the PI3K pathway accelerates degradation of cyclin D1 (64). PTEN-mediated inhibition of this pathway primarily decreased cyclin D3 levels in endometrial carcinoma cells, and enforced expression of cyclin D3 abrogated the cell cycle arrest in our study. Similarly, overexpression of cyclin D1 or CDK4 partially rescued PTEN-induced growth arrest of human keratinocytes (56), indicating that cyclin D can also modulate PTEN signaling in other cell types. In cycling cells, most $p27^{Kip1}$ is complexed with cyclin D-CDK4/6 (65, 66). In fact, the Cip/Kip proteins facilitate assembly and activation of cyclin D-CDK4/6 complexes (67). Alterations in the stoichiometry of components of the cyclin D-CDK4/6 complexes can shift the distribution of Cip/Kip inhibitors within the regulatory complexes. For example, in lung epithelial cells, addition of transforming growth factor-β did not change the level of $p27^{Kip1}$ but induced expression of $p15^{Ink4B}$. This change in stoichiometry of regulatory components resulted in a redistribution of $p27^{Kip1}$ from cyclin D-CDK complexes to cyclin E-CDK2 complexes and a cell cycle block (68). Similarly, the observed decrease in cyclin D3 in endometrial carcinoma cells observed here would decrease the available pool of cyclin D-CDK4/6 complexes and increase the pool of $p27^{KIP1}$ available to bind to CDK2. Therefore, $p27^{KIP1}$ remains an important effector of the PI3K-PTEN-AKT pathway, but its regulation is indirect. The role of $p27^{KIP1}$ in PTEN signaling is likely to be complex and dependent upon cell type and the context of the growth-regulatory signal.

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