PTEN Protects p53 from Mdm2 and Sensitizes Cancer Cells to Chemotherapy*

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The PTEN tumor suppressor protein inhibits phosphatidylinositol 3-kinase (PI3K)/Akt signaling that promotes translocation of Mdm2 into the nucleus. When restricted to the cytoplasm, Mdm2 is degraded. The ability of PTEN to inhibit the nuclear entry of Mdm2 increases the cellular content and transactivation of the p53 tumor suppressor protein. Retroviral transduction of PTEN into U87MG (PTEN null) glioblastoma cells increases p53 activity and expression of p53 target genes and induces cell cycle arrest. U87MG/PTEN glioblastoma cells are more sensitive than U87MG/PTEN null cells to death induced by etoposide, a chemotherapeutic agent that induces DNA damage. Previously, tumor suppressor proteins have been supposed to act individually to suppress cancers. Our results establish a direct connection between the activities of two major tumor suppressors and show that they act together to respond to stresses and malignancies. PTEN protects p53 from survival signals, permitting p53 to function as a guardian of the genome. By virtue of its capacity to protect p53, PTEN can sensitize tumor cells to chemotherapy that relies on p53 activity. p53 induces PTEN gene expression, and here it is shown that PTEN protects p53, indicating that a positive feedback loop may amplify the cellular response to stress, damage, and cancer.

The PTEN (1) tumor suppressor protein is a dual specificity phosphatase that dephosphorylates phosphatidylinositol 3-phosphate (PIP₂), a lipid second messenger, produced by activated phosphatidylinositol 3-kinase (PI3K) (2–4). PTEN mutants that retain protein tyrosine phosphatase activity but lose the ability to dephosphorylate PIP₂ are found in tumors, indicating that the lipid phosphatase activity of PTEN is required for its tumor suppressor activity (4, 5). Consistent with this conclusion, PTEN-deficient tumor cell lines and immortalized fibroblasts from PTEN−/− mice have elevated levels of PIP₂ (2, 4, 6). Once activated by mitogens or growth factors, including those produced by malignancies, PI3K induces activation of Akt/protein kinase B, a serine-threonine kinase that phosphorylates and inhibits pro-apoptotic BAD (7), caspase 9 (8), and FKHRL1 (9), a member of the forkhead family of transcription factors, and augments NF-κB activity, thereby promoting the induction of genes that suppress apoptosis (10–13). These observations indicate that PTEN functions as a tumor suppressor by inhibiting PI3K and Akt.

The p53 tumor suppressor protein is short-lived and non-abundant in normal cells (14, 15). In response to cell stress, p53 is activated and functions as a transcription factor to up-regulate gene products necessary for cell cycle arrest or apoptosis. Because stresses can be transient and need not irrevocably direct cells to a pathological course, the protein levels and transcriptional activity of p53 are regulated. The mdm2 proto-oncogene accomplishes both regulatory functions (16). The mdm2 gene is induced by p53, and the encoded protein binds the transcriptional activation domain of p53, thereby blocking recruitment of additional factors necessary for induction of gene expression (17–19). Once formed, the Mdm2/p53 complex shuttles from the nucleus to the cytoplasm (20–22) where Mdm2 targets p53 for degradation by acting as a ubiquitin ligase (23–25). Thus, p53 and Mdm2 form an autoregulatory feedback loop in which p53 positively regulates mdm2 expression and Mdm2 negatively regulates p53 (26). Thus, by binding the p53 protein, Mdm2 inhibits the transcriptional activity of p53, targets p53 for degradation, and prevents p53-dependent apoptosis (16–26).

PI3K/Akt signaling promotes translocation of Mdm2 from the cytoplasm into the nucleus where it negatively regulates p53 (27). Because PTEN inhibits PI3K signaling, we tested whether PTEN would block the action of Akt on Mdm2 and augment p53 activity. Demonstrating such a capacity would establish that tumor suppressors act coordinately and regulate one another’s functions, a major advance in our view of how these regulators of cell growth manifest their effects.

The present study shows that PTEN restricts Mdm2 to the cytoplasm and promotes Mdm2 degradation and p53 function. Thus, a connection between two major tumor suppressors has been discovered. A consequence of this relationship is that cancer chemotherapy that relies on p53 function is sustained in cells that contain functional PTEN and lost in PTEN null cells.
A new and important function for the PTEN tumor suppressor protein has been discovered.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture**—Stable clones of U87MG glioblastoma cells expressing wild-type or mutant PTEN were established under puromycin selection (2 μg/ml) (4). MDA 468 and MCF-7 cells, breast adenocarcinoma, T47D breast ductal carcinoma cells, 293T human embryonic kidney cells, U87MG glioblastoma cells, and H1299 non-small lung carcinoma cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C under 5% CO2.

**Confluent Microscopy**—Cells were transfected with FLAG PTEN or FLAG C124S using the calcium phosphate procedure. The transfected PTEN constructs were detected with anti-FLAG and a secondary antibody. Endogenous Mdm2 was detected with anti-Mdm2 antibody (IF-2) followed by a Cy5 secondary antibody. The nucleus was detected by staining with Syto 16 (Molecular Probes). Excitation of the stains was performed on a Bio-Rad MRC 1024 krypton/argon laser confocal imaging system under ×60 magnification and analyzed using the Metamorph imaging program. Consistent cytoplasmic or nuclear localization of Mdm2 was observed in the presence of the various constructs or treatment conditions in numerous fields.

**Gene Reporter Assays**—293 cells were co-transfected with RSV β-galactosidase and a p53 luciferase reporter construct containing the P2 promoter of p53 together with Mdm2, p53, PTEN, or combinations of these constructs using the calcium phosphate procedure. U87MG cells were transfected with a reporter composed of 15 copies of the p53-responsive element upstream of the luciferase gene and RSV β-galactosidase using LipofectAMINE. Gene reporter activity was then determined as described in the figure legends. Control experiments showed that Mdm2 and PTEN had no effect on RSV β-galactosidase or the p53 reporter.

**Cell Cycle Analysis**—U87MG or U87MG/PTEN cells were grown in medium containing 1% fetal bovine serum for 36 h. The cells were scraped into PBS, and 1 × 10^6 cells were washed twice with PBS and fixed in 40% ethanol. The cells were suspended in PBS containing 2.5 μg/ml propidium iodide and 50 μg/ml RNase, incubated at 37 °C for 30 min. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson). Cell fit analysis determined the percentage of cells in a specific stage of the cell cycle.

**Immunoprecipitation and Western Blot Analysis**—Cells were scraped into lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM sodium phosphate, 10 mM NaF, 1 mM sodium orthovanadate, 50 mM methylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin) and incubated on ice for 10 min. After centrifugation, 1 mg of the clarified cell lysate was incubated with 2 μg 1F-2 anti-Mdm2 for 1 h at which time 30 μl of a slurry of Protein A/G-agarose was added. After 1 h of incubation, the agarose was centrifuged, washed four times with ice-cold lysis buffer, suspended in Laemmli buffer, and boiled for 5 min. The immunoprecipitated proteins were fractionated on 8% polyacrylamide gels and transferred to Immobilon-P. Immunoblots were incubated with primary antibodies for 1–2 h at room temperature, washed, and incubated with a secondary antibody coupled to horseradish peroxidase for 1 h. Proteins were detected by chemiluminescence.

**Nuclear and Cytoplasmic Cell Fractions**—80% confluent cells were scraped into PBS and centrifuged. Nuclear and cytoplasmic fractions were isolated using the NE-PER kit (Pierce) according to the instructions of the manufacturer.

**MTT Assay**—U87MG, U87MG/PTEN, and U87MG/PTEN2 cells were plated in 96-well tissue culture plate. The next day, the amount of fetal bovine serum in the growth medium was reduced to 1%, and culturing continued in the absence or presence of various concentrations of etoposide or 10 μM LY294002. After 3 days MTT was added to a final concentration of 1 μM. After 4 h, the colored formazan crystals were dissolved in 100 μl of acidified isopropanol, and optical densities were read using a microplate reader at 570 nm. The absorbance values were corrected for blank readings. The absorbance at 570 nm directly corresponds with cell number. The percentage of cell survival was calculated as follows: (treated cells/untreated cell control) × 100. The data are plotted as drug concentration versus percentage of cell survival. Each data point is the average of results from six wells from two of each independent experiments.

**RESULTS**

**PTEN Regulates the Cellular Localization of Mdm2**—We anticipated that the ability of PTEN to inhibit PI3K/Akt signaling would restrict Mdm2 to the cytoplasm. Confocal microscopy shows that endogenous Mdm2 (stained in blue) is in the nucleus of growing cells transiently transfected with an inactive mutant of PTEN, C124S (stained in red) (Fig. 1a), but in the cytoplasm of cells transfected with wild-type PTEN. To demonstrate that PTEN was affecting Mdm2 localization by inhibition of PI3K activity, cells were treated with LY294002. This inhibitor of PI3K prevented movement of Mdm2 into the nucleus (Fig. 1b).

**PTEN Regulates Cellular Levels of Mdm2**—Mdm2 is a ubiquitin ligase, that, when localized to the cytoplasm, ligates ubiquitin to p53 and itself, thus targeting both proteins for proteasomal degradation (23–25). Transient transfection of PTEN with Mdm2 decreased Mdm2 protein levels compared with the level in p53 null H1299 cells transfected with Mdm2 (Fig. 2a). To extend the observations made with H1299 cells, LY294002, a PI3K inhibitor, was used to examine if blockade of PI3K signaling would lead to decreased levels of endogenous Mdm2. MCF-7 breast cancer cells were treated with LY294002 in the absence or presence of growth medium (serum) or IGF-1. As shown in Fig. 2b, LY294002 diminished the cellular content of Mdm2 in cells proliferating in growth medium or in cells treated with IGF-1. Furthermore, Mdm2 levels decreased in proliferating MDA 468, T47D, and 293 cells treated with LY294002, which abrogated PI3K activity and therefore activation of its downstream target, the Akt serine-threonine kinase (Fig. 2c). Thus, conditions that favor cytoplasmic localization of Mdm2, such as PTEN expression or inhibition of PI3K/Akt signaling downstream of PTEN, diminish Mdm2.

To more directly demonstrate that the effect of PTEN on Mdm2 levels resulted from an effect on PI3K/Akt signaling, 293 cells were co-transfected with FLAG-tagged Mdm2 together with HA-tagged CA-Akt or KD-Akt. Western blot analysis showed that the level of Mdm2 was greater in cells expressing CA-Akt than in those expressing KD-Akt (Fig. 3a). Thus conditions that favor cytoplasmic localization of Mdm2, such as PTEN expression or pharmacological inhibition of PI3K/Akt signaling downstream of PTEN, diminish Mdm2.

The half-life of Mdm2 is 20–40 min. Treatment of MCF7 cells with cycloheximide, a protein synthesis inhibitor, for 30 min showed that the half-life of Mdm2 was within the expected range in these cells. Pretreatment with LY294002 and then

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**FIG. 1. Effect of PTEN on Mdm2 localization.** Confocal analysis of Mdm2 localization in 293 cells: a, transiently transfected with FLAG-C124S (top) or FLAG-PTEN (bottom). FLAG-PTEN and FLAG-C124S (left) were detected with a FLAG antibody followed by a secondary Texas Red-conjugated antibody. Endogenous Mdm2 was detected with anti-Mdm2 for 1 h at which time 30 μM LY294002 (right) was detected with a FLAG antibody followed by a secondary Cy5 secondary antibody. The nucleus was detected with syto 16 (green) and Mdm2 with a FLAG antibody (blue).
cycloheximide greatly diminished Mdm2 levels (Fig. 3b), suggesting that cytoplasmic localization targets Mdm2 for degradation. To determine whether down-regulation of Mdm2 is mediated by proteasomal degradation, MCF-7 cells were treated with MG132, a proteasome inhibitor, or LY294002 and MG132. In each instance the amount of Mdm2 and ubiquitinated Mdm2 in the cells was increased by inhibition of the proteasome (Fig. 3c).

PTEN Promotes p53 Activity—We anticipated that ectopic expression of PTEN would up-regulate p53 activity and that catalytically inactive PTEN (C124S) would not. To test this supposition, p53 transactivation was measured using luciferase reporter constructs that contained deleted (mt) or wild-type (wt) p53 binding elements from the second mdm2 promoter upstream of luciferase. Transfection of PTEN into 293 embryonic kidney cells that contain wild-type p53 increased p53 reporter activity 4-fold, whereas C124S PTEN did not increase p53 activity (Fig. 4a). To analyze the ability of PTEN to block the ability of Mdm2 to suppress p53 activity, cells were transfected with p53 and Mdm2 together with varying amounts of PTEN. In the absence of PTEN, Mdm2 decreased p53 activity, and this effect was reversed in a dose-dependent manner by PTEN (Fig. 4b). Thus, a newly identified function of PTEN is to protect p53 from loss of function induced by Mdm2.

PTEN Promotes p53 Function and Sensitizes Glioblastoma Cells to Chemotherapy—The U87MG cell line, a human glioblastoma multiforme that contains wild-type p53 but is null for PTEN was transduced with a PTEN retroviral vector (4). Reconstitution was not toxic, because the level of PTEN expression achieved was not aberrant but, rather, was comparable to that in brain (4). Expression of PTEN in U87MG cells decreases tumor growth and increases the survival of mice implanted with these tumors (28). Thus, the U87MG/PTEN cell line, used in conjunction with the parental cell line, is a powerful model in...
which to study PTEN function.

We used the U87MG cell lines to define the PTEN/p53 relationship. To test the effect of the absence or presence of PTEN on p53 function in glioblastoma, gene reporter assays determined p53 activity in U87MG cells that did nor did not express PTEN. Because recent work has suggested that the mdm2 promoter can be regulated by factors other than p53 (29), experiments were also conducted with a synthetic p53 promoter composed of 13 copies of the p53-binding element upstream of the luciferase gene. In this way the effect of PTEN on p53 function was evaluated with two different promoters. PTEN expression in U87MG cells increased p53 luciferase activity (Fig. 5a). Consistent with the ability of PTEN to increase p53 activity, we observed increased expression of the p53 target genes, p21 and IGFBP3, in U87MG/PTEN cells relative to cells not expressing PTEN (Fig. 5b).

The results described above show that PTEN augments p53 activity and up-regulates p21, a cell cycle regulatory protein, whereas Mdm2 has the opposite effect. Thus, experiments were conducted to examine the effect of PTEN on the cell cycle. Over 36 h in 1% serum, U87MG cells proliferated, because 27% of the cells were in S phase. Under the same conditions, only 8% of the U87MG/PTEN cells were in S phase indicating diminished growth relative to U87MG cells (Fig. 6a).

PTEN inhibits the PI3K/Akt pathway that induces translocation of Mdm2 into the nucleus. This event permits interaction of Mdm2 with p53 and is succeeded by movement of p53-Mdm2 complexes into the cytoplasm where p53 is degraded. Based on the events just described, we tested the effect of PTEN expression on the localization of p53 and found p53 in the cytoplasm and nucleus of U87MG cells but in the nucleus of cells expressing PTEN (Fig. 6b). Consistent with our observation that PTEN augments the expression of p53 target genes (Fig. 5b), the Mdm2 protein level was higher in U87MG/PTEN than U87MG cells. Fig. 6b also shows that Mdm2 is predominantly in the nucleus of U87MG cells and in the cytoplasm of U87MG/PTEN cells. Thus, PTEN protects p53 by impairing movement of Mdm2 into the nucleus, which abrogates the mechanism responsible for loss of p53 and p53 function.

PTEN is mutated or deleted in 40–50% of high grade gliomas, which tend to be particularly refractory to chemotherapy (30). These clinical observations and the ability of PTEN to augment p53 activity suggested that introduction of PTEN into a null cell line would sensitize tumor cells to chemotherapeutic agents that induce DNA damage. In a survival assay, two clonal lines of U87MG cells stably expressing PTEN were tested for their sensitivity to etoposide. U87MG/PTEN cells express a 3-fold higher level of PTEN than the U87MG/PTEN2 cell line (28). Inhibition of PI3K signaling with LY294002, or expression of PTEN, sensitized cells to etoposide-induced death (Fig. 6c). Furthermore, cells expressing a higher level of PTEN (U87MG/PTEN) were more sensitive to low concentrations of etoposide than cells expressing a lower level of PTEN (U87MG/PTEN2). Thus, a consequence of the PTEN/p53 relationship is that the former tumor suppressor sensitizes cancers to DNA-damaging agents by blockade of PI3K/Akt/Mdm2 signaling, thereby permitting p53 to transmit its death signal.

DISCUSSION

The PTEN gene is frequently mutated in glioblastoma and in a significant fraction of prostate, endometrial, breast, lung, and other tumor types (30–32). Inherited germline mutations of PTEN are found in several rare autosomal dominant cancer predisposition syndromes, including Cowden's disease, Lhermitte-Duclos disease, and the Bannayan-Zonana syndrome (1, 33–36). The capacity to overcome mechanisms that promote
apoptosis is essential for the development and progression of cancer, and the PI3K/Akt pathway that PTEN negatively regulates provides such a mechanism by transmitting a strong survival signal. Activation of PI3K and Akt in response to nerve growth factor, insulin-like growth factor-1, platelet-derived growth factor, interleukin-3, and the extracellular matrix promotes cell survival (37–42). Thus, Akt activation can be necessary and sufficient for cell survival, a conclusion that emphasizes the significance of the negative regulatory effect of PTEN on this promiscuous survival factor.

p53 plays a major role in regulating the response of mammalian cells to stresses and damage, in part through the transcriptional activation of genes involved in cell cycle control, DNA repair, senescence, angiogenesis, and apoptosis (14, 15). Disruption of any of these processes can allow cells to escape from growth constraints, thereby permitting passage of mutations from one cell generation to the next thereby promoting the progression of cancer. Evidence for the central role of p53 as a tumor suppressor comes from genetic studies showing that mice homozygous for a deletion in the p53 gene develop tumors with high frequency (43, 44). Deletions or point mutations in the p53 gene are prevalent in the majority of human cancers (45, 46).

Five to ten percent of all human tumors overexpress Mdm2 as a consequence of gene amplification, increased transcription, or enhanced translation (16). Mdm2 overexpression is most often detected in soft tissue sarcomas, osteosarcomas, and in a high percentage of leukemias. A smaller subset of esophageal carcinomas, gliomas, anaplastic astrocytomas, and neuroblastomas overexpress Mdm2. Mdm2 overexpression is predictive of high grade, aggressive, metastatic malignancies that tend to be refractory to chemotherapy, leading to poor prognosis. Mdm2 has transforming potential, and the oncprotein appears to play an important role in carcinogenesis (16). Overexpression of Mdm2 immortalizes and transforms primary and continuously cultured cells (47, 48) and ectopic expression of an mdm2 transgene leads to spontaneous tumor formation in mice (49). Although a component of Mdm2 function may be independent of p53, the best-defined mechanism through which Mdm2 overexpression leads to uncontrolled cell growth is through inhibition of p53.

p53 controls the G, and G/M cell cycle checkpoints that mediate growth arrest (50). Mdm2 binds the N-terminal 42-amino acid transcriptional activation domain of p53 (51) and negatively regulates the transcriptional functions of p53, including its capacity to induce cell cycle arrest. One way that p53 controls the cell cycle is by induction of p21^WAF1 (52, 53), an inhibitor of cyclin-dependent kinases. PTEN protects the cell cycle regulatory function of p53 by blocking the ability of Mdm2 to impair induction of p21. Furthermore, PTEN is permissive of the induction of IGFBP3 by p53. This protein inhibits the capacity of IGF-1, which is produced by many tumor types, to activate cellular PI3K/Akt.

p53-induced cell death results from DNA damage or mutation that endangers an organism. The ability of p53 to induce death is antagonized by survival signaling, such as the PI3K/Akt signaling pathway, p53-mediated apoptosis in erythroblasts (54) and myeloid (55) cells is inhibited by interleukin-3, which delivers an anti-apoptotic signal through PI3K and Akt (39). In transformed baby rat kidney cells p53-mediated apoptosis is suppressed by oncogenic Ras, an activator of PI3K and Akt (56). A more direct link between p53 and PI3K and Akt is found in the observation that constitutively active PI3K and Akt delay the onset of apoptosis in cell cultures in which this event is dependent on p53 (57). Thus, PI3K/Akt signaling, as well as Mdm2, may affect p53-mediated apoptosis.

During the progression of cancer, malignant cells switch to an angiogenic phenotype (58). A consequence of this alteration is that production of inhibitors of angiogenesis down-regulates and/or production of inducers of angiogenesis up-regulates. Consequently, the effects of inducers of angiogenesis overwhelm the capacity of inhibitors to suppress neovascularization. Once tumors appropriate a blood supply from the host, they can grow aggressively and spread. PTEN suppresses tumor angiogenesis through an undescribed mechanism (28). A key suppressor of the angiogenic switch is p53 (58), which transcriptionally induces thrombospondin-1, a potent inhibitor of angiogenesis. The observations reported here, suggest PTEN suppresses angiogenesis by inhibiting of nuclear translocation of Mdm2, and thereby sustaining p53 activity.

Many drugs used to treat cancer directly or indirectly damage DNA, an event that triggers p53 activation (14, 15). Restoration of wild-type PTEN into tumors in which it is absent or mutated suppresses tumorigenicity, promotes apoptosis, and can induce chemosensitivity (59–61). High Mdm2 expression is found in aggressive, metastatic malignancies that tend to be refractory to chemotherapy, and such overexpression occurs in tumors in which mutations of p53 are absent (62, 63). The demonstration that PTEN negatively regulates the PI3K/Akt/Mdm2 survival pathway explains these observations.

Our data identify an important mechanism that leads to chemoresistance: The absence or mutation of PTEN leads to subsequent loss of the p53 protein and an inability of cells to respond to DNA-damaging agents with an apoptotic response. Our observations show that, by decreasing PI3K signaling, tumors can be sensitized to chemotherapy. The results presented here also show that the activity of Mdm2 is facilitated by inactivation of PTEN or enhanced PI3K/Akt signaling, which impair p53 function. Thus, PTEN is positioned to ensure that p53 can act as the gatekeeper and guardian of the genome (14). This conclusion shows that tumor suppressor proteins do not necessarily act individually to suppress inappropriate cell growth; rather, their functions can be concerted. Support for a strong symbiotic connection between p53 and PTEN is found in the demonstration that p53 promotes PTEN transcription (64), and here it is shown that PTEN sustains p53 function. Thus, cells may respond to stresses, damage, and cancers byactivation of a positive feedback loop that promotes apoptosis.

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