Nuclear-Cytoplasmic Partitioning of Phosphatase and Tensin Homologue Deleted on Chromosome 10 (PTEN) Differentially Regulates the Cell Cycle and Apoptosis

Ji-Hyun Chung and Charis Eng

1,2,3,4,5,6

Abstract

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a tumor suppressor phosphatase that dephosphorylates both protein and lipid substrates, is found to be mutated in both heritable and sporadic breast cancer. Cellular PTEN has been shown to regulate Akt phosphorylation, mitogen-activated protein kinase (MAPK) phosphorylation, p27Kip1, and cyclin D1 protein levels. Additionally, we and others have shown that PTEN can regulate not only the cell cycle but also cellular apoptosis. Until recently, the functions of PTEN have been thought to occur through cytoplasmic PTEN. However, we have shown that PTEN localizes to the nucleus and that this localization coincides with the G0-G1 phases of the cell cycle. Furthermore, we have shown that PTEN has bipartite nuclear localization sequence (NLS)-like sequences that are required for major vault protein-mediated nuclear import. These findings suggest that subcellular localization of PTEN may regulate its function and that nuclear-localized PTEN may regulate unique cellular functions that have been attributed to cytoplasmic PTEN. To examine this possibility, we analyzed downstream PTEN readouts using MCF-7 Tet-Off breast cancer cell lines stably transfected with two different NLS mutant PTEN constructs, which do not localize to the nucleus, and compared these with cells transfected with wild-type PTEN and empty vector control cells. We found that cytoplasmic PTEN down-regulates phosphorylation of Akt and up-regulates p27Kip1, whereas nuclear PTEN down-regulates cyclin D1 and prevents the phosphorylation of MAPK. Additionally, whereas we observe that nuclear PTEN is required for cell cycle arrest, we found that cytoplasmic PTEN is required for apoptosis. Our observations show that nuclear-cytoplasmic partitioning differentially regulates the cell cycle and apoptosis and, in this manner, provide further evidence that nuclear import of PTEN should play a role in carcinogenesis. (Cancer Res 2005; 65(18): 8096-100)

Introduction

An important function of activated phosphatidylinositol triphosphate kinase (PI3K) in cells is the inhibition of programmed cell death or apoptosis, and Akt is a good candidate for mediating these PI3K-dependent cell survival responses. Akt has been implicated as an antipoptotic in many different cell death paradigms and the crosstalk between Akt and phosphatase and tensin homologue deleted on chromosome 10 (PTEN) extends to many levels that includes both Akt activators and Akt targets, making this interaction extremely interesting and complex (1). The tumor suppressor gene encoding PTEN, a dual-specificity phosphatase, is somatically mutated and/or deleted in a wide variety of diverse human cancers, including carcinomas of the breast, endometrial, prostate, and glioblastoma. Germ line mutations in PTEN have been found in the dominantly inherited Cowden and Bannayan-Riley-Ruvalcaba syndromes, which are characterized by multiple hamartomas and an increased risk of malignant and benign breast, thyroid, and endometrial tumors (2). The many important roles of PTEN span a diverse range of biological processes, including G1 cell cycle arrest, apoptosis, cell migration inhibition, spreading, chemotaxis, and focal adhesion formation (3). PTEN is a lipid phosphatase that dephosphorylates phosphatidylinositol-(3,4,5)-triphosphate [P(3,4,5)P3]. P(3,4,5)P3 is a lipid second messenger and a regulator of the PI3K/Akt pathway (4). The lipid phosphatase activity of PTEN has been shown to regulate Akt phosphorylation as well as p27Kip1 protein levels. P(3,4,5)P3 is required for Akt recruitment to the plasma membrane and its subsequent activation, whereas PTEN inhibits the proliferative Akt-dependent pathways (1). Thus, PTEN leads to decreased phosphorylated-Akt (P-Akt) levels and induces apoptosis. PTEN dephosphorylates tyrosine-, serine-, and threonine-phosphorylated peptides in vitro and has been shown to dephosphorylate focal adhesion kinase in vivo (3). In addition, the protein phosphatase activity of PTEN is known to regulate mitogen-activated protein kinase (MAPK) phosphorylation and cyclin D1 protein levels and these activities have been assumed to be due to the actions of PTEN in the cytoplasm. We previously showed that PTEN localizes to the nucleus and this localization occurs with the G0-G1 phases of the cell cycle (5). In addition, we showed that PTEN has nuclear localization sequence (NLS)-like sequences that mediate nuclear import (6). Recently, it was shown that PTEN induces cell cycle arrest by decreasing the levels and nuclear localization of cyclin D1 (7). The regulated movement of the tumor suppressors between nucleus and cytoplasm provides an efficient, simple, and rapid way for tumor suppressors to control cell growth. Indeed, during recent years, several known tumor suppressors have been shown to undergo nuclear-cytoplasmic shuttling (8). It has been documented that Merlin accumulates perinuclearly at the G2-M phase and shifts to the nucleus at early G1 (9). A similar pattern of expression has also been shown for the adenomatous polyposis
coli and von Hippel-Lindau tumor suppressors, which localize mostly in the cytoplasm of confluent cells and shift to the nucleus in the subconfluent cells and affect growth in a cell cycle–dependent manner (10). Hence, we broadly hypothesized that the various functions of PTEN and PTEN-like dual-specific tumor suppressor proteins may compartmentalize for their different tumor-suppressive effects (11). Whereas the role of cellular, often assumed cytoplasmic, PTEN in regulating apoptosis and the cell cycle has been under intense scrutiny, little is known about the effects, if any, of nuclear PTEN on cellular signaling events. Therefore, we investigated the function of nuclear PTEN and compared it with that of cytoplasmic PTEN with an inducible system consisting of two nuclear PTEN localization-deficient cell lines that we established for nuclear localization studies. We are able to show that PTEN-induced cell cycle arrest is mediated by nuclear PTEN, whereas cytoplasmic PTEN induces apoptosis. Our findings provide insights into the molecular mechanism by which the dual location of PTEN dictates specific function in tumor progression.

Materials and Methods

Cell lines and culture conditions. The MCF-7 breast cancer cells expressing wild-type PTEN (PTEN:WT), PTEN:M2M4, and PTEN:M3M4 were generated and maintained as previously described (6). In brief, four sequences in PTEN were selected as NLS-like sequences due to similarity to known nontraditional NLS and mutant constructs were generated using PCR-directed mutagenesis to incorporate mutations in the regions encompassing the putative NLS sequences. The constructs were stably transfected into the MCF-7 Tet-Off cell line stably expressing nuclear localization-defective PTEN mutants (PTEN:M2M4 and PTEN:M3M4) as well as wild-type PTEN and vector control cells. Vector expression was controlled with 2 μg/mL Tet. Cells were synchronized with 3 mmol/L hydroxyurea for 16 to 24 hours after Tet removal for 24 hours.

SDS-PAGE, Western blot, and antibodies. Protein extraction and immunoblotting were done as previously described (6). Whole cell, cytoplasmic, and nuclear fractions were isolated, separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The monoclonal antibody 6H2.1 raised against the COOH terminal of PTEN (Cascade Biosciences, Co., Portland, OR; ref. 10) was used for PTEN detection (12). Antibodies against P-MAPK, MAPK, Akt, P-Akt, and actin were purchased from Cell Signaling, Co. (Beverly, MA). Antibodies against p27kip1, p21cip1, cyclin D1, and cyclin E were purchased from Santa Cruz, Co. (Santa Cruz, CA).

Cell growth assay and cell cycle experiments. For cell growth, equal number (2 × 10^5) of control cells and those stably expressing either PTEN or NLS-mutated PTEN were plated in six-well plates. After 48 hours, the medium was removed and the cells were washed and replaced with media ± Tet. At the indicated times, cells were harvested by trypsinization and viable cells (excluding trypan blue) were counted. For cell cycle analysis, cells were synchronized with 3 mmol/L hydroxyurea for 16 to 18 hours and then released from hydroxyurea arrest by replacement of media ± Tet. At each time point (0, 4, 8, 12, and 24 hours), cells were harvested, resuspended in ice-cold 70% ethanol, and stored at −20°C until further analysis. Washed cells were stained with 1 μg/mL propidium iodide (Sigma Chemical, Co., St. Louis, MO) in 0.1% Triton X-100 in PBS. Flow cytometry was done using a Beckman-Coulter elite flow cytometer with a 610 log pass filter for data collection. Data were filtered and cell cycle phases were quantified using the ModFit Program (Verify Software, Bowdoin, ME).

Caspase-3/7 activity assay. Apoptosis assays were done using CV-Caspase 3/7 detection kit as recommended by the manufacturer (Biomol, Inc., Plymouth Meeting, PA). Equal number of cells was plated into Tet media and synchronized by hydroxyurea as described above. Cells were released from hydroxyurea arrest by replacement of media ± Tet. At each time point, cells were collected and analyzed.

Results

Cytoplasmic PTEN down-regulates the PI3K/Akt pathway and up-regulates p27kip1. Our previous data showing nuclear PTEN peaking with G0-G1 of the cell cycle suggested that subcellular localization of PTEN may play a role in regulating its downstream outcomes (5). To examine this possibility, we used MCF-7 Tet-Off breast cancer cell lines stably expressing two different nuclear localization defective PTEN mutants (PTEN:M2M4 and PTEN:M3M4, which we will collectively refer to as NLS-PTEN mutants) as well as wild-type PTEN and empty vector control cells. We have previously shown that PTEN:M2M4 and PTEN:M3M4 do not localize to the nucleus, whereas wild-type PTEN does (Fig. 1; ref. 6).

We and others have shown that the lipid phosphatase activity of PTEN is involved in the down-regulation of P-Akt and the increase in p27kip1 levels (13); however, the role of cytoplasmic versus nuclear PTEN in this regulation is not known. We show here that cellular P-Akt protein levels decreased and p27kip1 protein levels increased in cells expressing wild-type PTEN as previously described (Fig. 1B; ref. 13). NLS-PTEN mutants also showed a decrease in P-Akt levels and an increase in p27kip1 levels (Fig. 1B). When we examined P-Akt and p27kip1 levels in subcellular fractions, we found P-Akt levels decreased in nuclear and cytoplasmic...
Nuclear PTEN down-regulates the phosphorylation of MAPK. Whole cell extracts (A) and nuclear-cytoplasmic fractions (B) were prepared as described in Materials and Methods and examined by immunoblotting for P-MAPK (open columns) and MAPK (tiled columns) protein levels in the absence of tetracycline. Note that nuclear PTEN overexpression down-regulates the phosphorylation of MAPK in whole cell extracts (P < 10⁻⁵) and cytoplasmic fractions (P < 10⁻⁵). Data in (A) and (B) were quantified by phosphoimage analysis and normalized to nuclear and cytoplasmic fractions in vector-only cell lines. Statistical analysis was done using an unpaid (equal variance) t-test. Each group was compared with a vector control; P < 0.05 is considered significant. Columns, mean (n = 3); bars, SD.

We have shown that PTEN decreases cyclin D1 levels via its protein phosphatase activity (15). As shown in Fig. 3, the overexpression of wild-type PTEN results in decreased cyclin D1 levels in whole cell extracts (P < 10⁻⁵). This is due to a decrease in cyclin D1 levels in both nuclear and cytoplasmic fractions (P < 10⁻³ and <10⁻⁴, respectively; Fig. 3B). In contrast, we found that when cells were induced to express the NLS-PTEN mutants, the levels of cyclin D1 were not altered. These results indicate that nuclear localization of PTEN is required for its down-regulation of cyclin D1. Interestingly, wild-type PTEN overexpressed cells showed a 15% decrease in nuclear cyclin D1 compared with other cells (Fig. 3B).

Nuclear PTEN induces G₀-G₁ cell cycle arrest and cytoplasmic PTEN induces apoptosis. To further examine the role of nuclear PTEN on the cell cycle, growth, and apoptosis, cells were synchronized with hydroxyurea, subsequently released, and harvested at each time point. As expected, cells overexpressing wild-type PTEN grew slowly compared with control cells. In contrast, cells overexpressing either NLS-PTEN mutant did not show this inhibition of cell growth (Fig. 4A). This suggests that nuclear-localized PTEN is responsible for the inhibition of cell growth. To further examine the growth inhibition, cells were synchronized with hydroxyurea and analyzed using flow cytometry. On average, 70% of each of the four cell lines were in G₀-G₁ at the time of hydroxyurea release, reached S phase by 4 to 8 hours, and returned to 60% G₀-G₁ by 24 hours (Fig. 4B). In spite of this similar starting profile, wild-type PTEN-expressing cells had more cells remaining in G₀-G₁ after hydroxyurea removal. Cells that overexpressed wild-type PTEN showed the highest level of G₀-G₁ arrest at 4 hours.
Activation of caspases is a common distal event of caspase-mediated apoptosis. To determine whether nuclear or cytoplasmic PTEN regulates apoptosis, we examined the effect of nuclear and cytoplasmic PTEN on cell death by determining caspase-3/7 activity in cells expressing wild-type or NLS-PTEN. Induction of PTEN expression for 48 hours led to a 15% increase in caspase-3/7 activity in both wild-type and NLS-PTEN mutant cells but not in control cell lines (Fig. 4C). This indicates that cytoplasmic, but not nuclear PTEN regulates apoptosis.

Discussion

It is well established that PTEN regulates cell growth and cell cycle arrest but whether compartmentalized PTEN plays any role was previously unknown (3). Whereas the role of PTEN has been established in the cytoplasm as a phosphatase, we and others first noticed the possibility of PTEN existing in the nucleus when we did immunohistochemistry on breast, thyroid, and endocrine pancreatic tumors and cutaneous melanomas (2). We have recently shown that PTEN has dual NLS-like sequences that mediate nuclear import (6). Despite these hints of the presence of nuclear PTEN, little is known about the mechanism of PTEN localization or its role in the nucleus. We have shown here that nuclear PTEN is required for PTEN-mediated cell cycle arrest and growth inhibition via down-regulation of MAPK and cyclin D1, whereas cytoplasmic PTEN is sufficient for PTEN-mediated down-regulation of Akt phosphorylation and up-regulation of p27Kip1 levels and is required for caspase-mediated apoptosis.

It is well known that cytoplasmic PI(3,4,5)P3 promotes cell survival (3). Recently, it has been reported that nuclear PI(3,4,5)P3 promotes cell survival by binding to nucleolar phosphoprotein B23 (16), which suggested that PTEN could operate as a key regulator for cell survival in both the nucleus and cytoplasm. Notably, nuclear PTEN expression was strong in normal cells and seemed to wane (and enter the cytoplasm) in cancerous counterparts, suggesting that nuclear PTEN is required for the tumor-suppressing ability of PTEN (17). Our current observations support the concept that proper nuclear localization and function of PTEN is critical for cellular homeostasis and, at a minimum, mediates cyclin D1-mediated G1 cell cycle arrest.

Cyclin D1, a key regulator of G0-G1 cell cycle progression, accumulates in the nucleus throughout the G1 phase (18). Cyclin D1 localizes to the nucleus in the G1 phase and exits the nucleus as cells progress through the S phase (19) and PTEN prevents its nuclear localization (7). Our observations are, therefore, of interest: nuclear PTEN decreases the levels and nuclear localization of cyclin D1 (Figs. 3A and B). Also, nuclear and cytoplasmic cyclin D1 were shown to decrease in wild-type PTEN cells along with a decrease in phosphorylation of MAPK (Fig. 2). This may suggest that MAPK regulation by nuclear PTEN might control the levels of cyclin D1 (Figs. 2 and 3) such that decreased nuclear cyclin D1 decreases the levels and nuclear localization of cyclin D1 (Figs. 3A and B). This is tantalizing to postulate that the protein phosphatase activity of PTEN is more active, or pertinent, in the nucleus, and that nuclear PTEN has tumour-suppressing ability via cell cycle arrest. Our studies, therefore, support our hypothesis and identify nuclear PTEN as necessary for cell cycle arrest.

PTEN coordinates G1 arrest by down-regulating cyclin D1 protein levels via its protein phosphatase activity and up-regulating p27Kip1 levels via its lipid phosphatase activity (13). Increased levels of CDK inhibitors seem to mediate cell cycle arrest (15). When
p21^{kip1} and p27^{kip1} levels were examined in subcellular fractions, we found that p27^{kip1} levels increased in both nuclear and cytoplasmic fractions in cells expressing either wild-type PTEN or NLS-mutated PTEN but the level of p21^{kip1} remained the same in all four cell lines (Fig. 1B). Thus, up-regulation of p27^{kip1} by PTEN might depend on the actions of PTEN in the nucleus, and that nuclear PTEN is not germane in mediating the CDK inhibitors.

Because PTEN is predominantly located in the cytoplasm, it may be logical to conclude that cytoplasmic PTEN controls the apoptotic pathway through dephosphorylation of P(3,4,5)P3, which recruits Akt to the cellular membrane where Akt is phosphorylated and activated by upstream kinases (20). However, the role of subcellular localization of PTEN as a means of regulating downstream signals has not been investigated. Here, we have observed that phosphorylation of Akt in nuclear fractions decreased in cells carrying PTEN nuclear localization–defective mutants as well as wild-type PTEN cells (Fig. 1). Therefore, we presume that Akt may transport through the nuclear membranes and that the lipid phosphatase activity of PTEN is either more active or more important in the cytoplasm.

Superficially, our current data may be puzzling in the context of the immunohistochemical observations that expression of PTEN in the nucleus and cytoplasm is high in normal cells but nuclear PTEN wanes with maintenance of cytoplasmic PTEN in malignant cells (16). Because we have shown cytoplasmic PTEN to be required for apoptosis, it is surprising that it would be insufficient to suppress neoplastic proliferation. There is currently no conclusive explanation, but we may postulate that the fine balance of the lipid phosphatase activity of PTEN and protein phosphatase activity in a compartment-specific manner may be the sentinel decision point between neoplastic proliferation and normal cell cycling and apoptosis. Further, PTEN has other functions beyond cell cycle control and apoptosis regulation: It is possible that nuclear versus cytoplasmic PTEN may influence cell migration and invasion. These possibilities remain to be investigated.

In summary, we have presented a molecular mechanism by which the dual localization of PTEN—cytoplasm and nucleus—dictates specific function in tumor progression. Interestingly, our data suggest that lipid phosphatase activity of PTEN may have a more dominant role in the cytoplasm whereas the protein phosphatase activity of PTEN may predominate in the nucleus. Our current findings provide insights into the mechanism by which the nuclear-cytoplasmic partitioning of PTEN can differentially regulate the cell cycle and apoptosis. Nuclear-cytoplasmic compartmentalization of tumor suppressors may be a nontraditional mechanism for separating the multifunctions of these molecules and may serve as a novel mechanism for targeting therapy. Thus, our data now shift us away from the dogma of cytosplamic PTEN signaling, and into the world of nuclear PTEN signaling and how the two interact.

Acknowledgments

Received 6/1/2005; revised 7/20/2005; accepted 7/27/2005.

Grant support: American Cancer Society grant RSG-02-151-01-CCE, Susan G. Komen Breast Cancer Research Foundation grant BCTR 2000 462 (both to C. Eng), and the National Cancer Institute grant P50CA16658 (to the Comprehensive Cancer Center).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Michelle Sinden and Dr. Kristin Waite for helpful discussions, and Dr. Waite for critical review of multiple drafts of the manuscript.

References

Nuclear-Cytoplasmic Partitioning of Phosphatase and Tensin Homologue Deleted on Chromosome 10 (PTEN) Differentially Regulates the Cell Cycle and Apoptosis

Ji-Hyun Chung and Charis Eng


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/18/8096

Cited articles
This article cites 19 articles, 11 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/18/8096.full.html#ref-list-1

Citing articles
This article has been cited by 30 HighWire-hosted articles. Access the articles at:
/content/65/18/8096.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.