Functional Evaluation of PTEN Missense Mutations Using in Vitro Phosphoinositide Phosphatase Assay

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Abstract
The tumor suppressor gene PTEN is frequently mutated in diverse human cancers and in autosomal dominant cancer predisposition disorders. Recent studies have shown that the lipid phosphatase activity of PTEN is critical for its tumor suppressor function and that PTEN negatively regulates the phosphatidylinositol 3-kinase-protein kinase B pathway. Although more than half of PTEN mutations result in protein truncation, a significant fraction of PTEN mutations are missense mutations. To examine whether tumor-derived and germ-line-derived missense mutations inactivate PTEN lipid phosphatase function, we constructed 42 distinct types of PTEN missense mutations and expressed them in Escherichia coli. The purified (His)_8-tagged PTEN proteins were tested for their ability to dephosphorylate inositol 1,3,4,5-tetrakisphosphate and phosphatidylinositol 3,4,5-triphosphate. In addition, we examined the effect of mutant PTENs on the ability of PTEN to bind to the phospholipid membrane. The results revealed that the majority of PTEN missense mutations [38 of 42 (90%)] eliminated or reduced phosphatase activity and that all of the mutations examined had no effect on the membrane binding activity of PTEN. Our study indicated that phosphoinositide phosphatase activity is important for the tumor suppressor function of PTEN and that there may be other mechanisms of PTEN inactivation that are not monitored by in vitro phosphatase assay and in vitro membrane binding assay.

Introduction
The PTEN/MMAC1/TEP1 gene (referred to hereafter as PTEN) was identified recently as a putative tumor suppressor gene located on human chromosome 10q23.3 (1–3). Somatic deletions or small mutations of PTEN have been observed with high frequency in malignant glioma (4, 5) and endometrial cancer (6, 7) and at a lower rate in other malignancies such as prostate cancer (8), small cell lung cancer (9), melanoma (10), hepatocellular carcinoma (11), and breast cancer (12). Germ-line mutations of PTEN have been detected frequently (23–25) in the autosomal dominant hamartoma cancer syndromes Cowden disease (OMIM153480), Bannayan-Zonana syndrome (OMIM153830), and Lhermitte-Duclos disease (13, 14). In a murine model, heterozygous knockout mice develop tumors in multiple organs; whereas homozygous deletion of PTEN is embryonically lethal (15, 16). Furthermore, enforced expression of PTEN cDNA inhibits cell migration and spreading (17, 18) and suppresses tumor cell growth by arresting the cell cycle at G_1 phase and/or by inducing apoptosis (19). This genetic and biological evidence suggests that PTEN has an important function in tumorigenesis as well as in normal embryonic development.

The PTEN gene contains nine exons and encodes a 403-amino acid cytoplasmic protein showing extensive NH_2-terminal homology with tensin and auxillin as well as having a central catalytic domain showing perfect homology with PTP and dual-specific phosphatases (1–3). A key step in understanding the function of PTEN as a tumor suppressor is to identify its physiological substrates. Data thus far suggest that PTEN possesses two distinct phosphatase activities. One is a phosphoinositide phosphatase activity against phosphoinositides, such as Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3, which involves dephosphorylation of the D3-position phosphate of the inositol ring (20). The other is protein phosphatase activity, including focal adhesion kinase (21). Several studies have shown that the phosphoinositide phosphatase activity of PTEN is critical for its tumor suppressor function, whereas activity toward the protein substrate is not essential for growth suppression (18, 22–25). This suggests that the former activity may play a more important role in tumor suppression by PTEN. Because PtdIns(3,4,5)P_3 is a phospholipid second messenger produced by PI3K, PTEN seems to be involved in the PI3K signaling pathway (26). One of the important downstream targets of PI3K is protein kinase B/Akt, which controls cell proliferation and protects cells from apoptosis (27). The fact that both PI3K and Akt play critical roles in a variety of growth factor signaling pathways raises the possibility that PTEN functions as a tumor suppressor through negative regulation of the PI3K/Akt pathway.

Loss of PTEN function can occur through homozygous gene deletion, point mutation plus loss of the remaining allele, or loss of expression (1, 2, 4, 28). We reviewed both somatic and germ-line PTEN mutations by surveying a database of the published literature. There were about 417 mutations reported by the end of August 1999. Among them, 143 (34%) were missense mutations including 90 distinct types, 162 (39%) were frameshift mutations, 73 (18%) were nonsense mutations, and 39 (9%) were other mutations containing in-frame deletion/insertion or splicing site mutations. The frequency of missense mutations in the PTEN gene was higher than that in other tumor suppressor genes except for the p53 gene, which had the highest frequency of missense mutations (~90%). Although both truncating and missense PTEN mutations were scattered throughout the nine exons of PTEN, the missense mutations tended to cluster around the phosphatase domain. In fact, several studies showed that a limited number of reported missense mutations within the phosphatase domain could abrogate the protein phosphatase and/or phosphoinositide phosphatase activity of PTEN (22, 25). Even so, the majority of missense mutations, including mutations outside the phosphatase do-

Received 3/1/00; accepted 5/4/00.
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3 The abbreviations used are: PTP, protein tyrosine phosphatase; Ins(1,3,4,5)P_4, inositol 1,3,4,5-tetrakisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; PI3K, phosphatidylinositol 3'-kinase; LMV, large multilamellar vesicle; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

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main, remain to be analyzed. In general, analyses of the effects of missense mutations derived from a disease on the functional properties of the gene product are advantageous to elucidate the most important function for pathogenesis. Therefore, we evaluated the pathogenic effect of a series of PTEN missense mutations.

In this study, we constructed 42 distinct missense mutations that mapped within the PTEN open reading frame and examined the phosphoinositide phosphatase activity of the (His)_{6}-tagged PTEN protein corresponding to each point mutation against Ins(1,3,4,5)P_{4} and PtdIns(3,4,5)P_{3} as well as their ability to bind a phospholipid membrane in vitro.

Materials and Methods

Vector Construction and Cloning of Mutant PTEN Alleles. The wild-type histidine-tagged PTEN ([His]_{6}-PTEN) expression vector was constructed as follows. The full-length open reading frame of PTEN cDNA was amplified by PCR using Pfu DNA polymerase (Stratagene, La Jolla, CA) and a pair of primers with BamHI and HindIII sites. The restriction endonuclease-treated PCR product was inserted into the BamHI/HindIII sites of the pQE30 vector (Qiagen, Hilden, Germany). PTEN cDNA with missense mutations (S10N, Y16C, G20E, Y27S, L42R, H61R, Y68H, C71Y, H93Y, C105F, D107Y, C136Y, Y155C, G165R, S170N, S170R, R173C, L345Q) were present. These vectors are identical to the wild-type (His)_{6} -PTEN, the vector was transformed into E. coli strain M15 harboring pREP4 (Qiagen). The resulting transformant was cultured in 50 ml of Luria-Bertani medium at 37°C by mid-log phase (A_{600 nm} = 0.6). Isopropyl β-D-thiogalactopyranoside was added at a concentration of 0.2 mM, and the culture was incubated for an additional 6 h at 25°C. Thereafter, all of the procedures were performed at 4°C. The bacterial cells were harvested by centrifugation, and the bacterial pellet was frozen at −80°C until use. The frozen pellets were resuspended in 1 ml of ice-cold lysis buffer containing 50 mM NaH_{2}PO_{4} (pH 8.0), 500 mM NaCl, 5 mM imidazole, 5 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, and bacteriolysis was performed by sonication until the cell suspension became transparent. After the addition of 10 mM phenylmethylsulfonyl fluoride by using Nanosep (Pall Filtron, Northborough, MA) and centrifuged to a volume of 50 ml of TED buffer (50 mM HEPES-KOH (pH 7.2), 100 mM NaCl by sonication and collected by centrifugation. An aliquot of (His)_{6}-PTEN protein (2–5 μg) and 100 μl of LMVs were incubated in 50 mM HEPES-KOH (pH 7.2) and 2 mM CaCl_{2} for 15 min at 25°C. After centrifugation at 12,000 × g for 10 min at 4°C, the pellets containing lipid and bound proteins were washed in 500 μl of the above-mentioned buffer and dissolved in SDS sample buffer. The amount of free phosphate was determined by measuring the A_{620 nm}. A standard curve was generated in each assay, and the amount of free phosphate was calculated from the standard curve line-fit data.

Phospholipid Binding Assay. The PTEN protein binding to LMVs consisting of three different phosphoglycerides, PS, PE, and PC (Sigma, St. Louis, MO), was carried out according to the procedures published previously (31–33), with some modifications. Briefly, 100 μg of LMVs (PS:PE:PC ratio, 35:50:15) were prepared in 10 ml of buffer containing 50 mM HEPES-KOH (pH 7.2) and 100 mM NaCl by sonication and collected by centrifugation. An aliquot of (His)_{6}-PTEN protein (2–5 μg) and 100 μl of LMVs were incubated in 50 mM HEPES-KOH (pH 7.2) and 2 mM CaCl_{2} for 15 min at 25°C. After centrifugation at 12,000 × g for 10 min at 4°C, the pellets containing lipid and bound proteins were washed in 500 μl of the above-mentioned buffer and dissolved in SDS sample buffer. The addition of an equal volume of 20% (w/v) trichloroacetic acid precipitated the proteins in the supernatant. After a 15-min incubation on ice, the samples were centrifuged at 12,000 × g for 15 min at 4°C, washed twice with acetone, and mixed with SDS sample buffer. Equal proportions of the supernatants and pellets were analyzed by 10% (w/v) SDS-PAGE followed by silver staining (Daiichi Pure Chemicals, Tokyo, Japan).

Results

To examine whether the missense mutation inactivates the phosphoinositide phosphatase function of PTEN, we selected 42 distinct tumor-derived or germ-line-derived missense mutations covering more than 40% of the reported missense mutation types. Each mutation was constructed by site-directed mutagenesis, expressed as a (His)_{6}-tagged fusion protein ([His]_{6}-PTEN) in E. coli, and purified as described in “Materials and Methods.” The amount of expressed (His)_{6}-PTEN was measured by spectrophotometer and confirmed by SDS-PAGE. The representative data are shown in Fig. 1. To assess phosphoinositide phosphatase activity, we used two phosphoinositide substrates with [PtdIns(3,4,5)P_{3}] or without [Ins(1,3,4,5)P_{4}, a diacylglycerol chain. Bacterially expressed PTEN has been shown to dephosphorylate the phosphate in the D3 position of the inositol ring in each substrate (20, 30, 33).

Phosphatase Activities of PTEN against Ins_{1,3,4,5}P_{4}. To examine whether the missense mutations inactivate normal PTEN phosphoinositide phosphatase activity, the His_{6}-tagged PTEN protein used for the PtdIns(3,4,5)P_{3} phosphatase assay was extracted again as described above, except that NaH_{2}PO_{4} was replaced with 50 mM Tris-HCl (pH 8.0) in the buffer used for lysis, washing, and elution. The phosphatase assays (30) were performed in 50 μl of reaction buffer containing 100 mM Tris-HCl (pH 8.0), 10 mM DTT, 200 μM water-soluble D_{i}C_{60}-PtdIns(3,4,5)P_{3} (Echelon, Salt Lake City, UT), and 2 μg of (His)_{6}-PTEN protein at 37°C for 40 min. The phosphate released from the substrate was measured using Green Reagent (Biomol, Plymouth Meeting, PA) according to the manufacturer’s instructions. In brief, 500 μl of Green Reagent were added, followed by incubation for 20 min at room temperature. The concentration of the released phosphate was determined by measuring the A_{620 nm}. A standard curve was generated in each assay, and the amount of free phosphate was calculated from the standard curve line-fit data.

### Functional Evaluation of PTEN Missense Mutations

**Fig. 1.** Bacterial expression and purification of PTEN. Recombinant (His)_{6}-PTEN expressed from wild-type PTEN, null pQE30 vector, and mutant PTEN (Y68H, S170N, G251C, K299E, D331G, L345Q) in E. coli was purified using Ni-NTA-agarose, as described in “Materials and Methods.” Approximately 0.5–1 μg of the proteins was separated by SDS-PAGE and visualized by Coomassie Blue staining. BSA was used for judging the concentration of the PTEN protein. N Dal, null pQE30 vector as a negative control; PTEN, wild-type PTEN as a positive control.
phatase activity against Ins(1,3,4,5)P_4, (His)_6-PTEN harboring the indicated amino acid substitutions was assayed for its ability to dephosphorylate the phosphate in the D3 position of Ins(1,3,4,5)P_4 using the method described previously (Ref. 20; Fig. 2). The results showed that four mutant PTENs (9%; S10N, L42R, V369G, and T401I) retained activity comparable with or even higher than that of normal PTEN, and seven mutant PTENs (17%; G20E, M134L, S227F, K289E, D331G, K342N, and F347L) retained partial activity (Fig. 2). The remaining 31 mutant PTENs (74%) had no activity.

Phosphatase Activities of PTEN against PtdIns(3,4,5)P_3. Because Ins(1,3,4,5)P_4 lacks the diacylglycerol chain of PtdIns(3,4,5)P_3, we compared the phosphoinositide phosphatase activities of 12 selected mutant PTENs and normal PTEN toward the two substrates. These PTENs contained mutants with full (S10N, L42R, V369G, and T401I), partial (G20E, M134L, S227F, and K342N), and no (Y68H, G129E, R130G, and R173C) phosphatase activities against Ins(1,3,4,5)P_4. Water-soluble PtdIns(3,4,5)P_3 was used to examine the released phosphate. The eight mutant PTENs with full or partial Ins(1,3,4,5)P_4 phosphatase activity showed nearly the same level of phosphatase activity against PtdIns(3,4,5)P_3 as wild-type PTEN, whereas the four mutant PTENs with no Ins(1,3,4,5)P_4 phosphatase activity also eliminated PtdIns(3,4,5)P_3 phosphatase activity (Fig. 3). These results indicated that the effects of the missense mutations on PTEN phosphatase activity against the two phosphoinositides were nearly identical, although the assay using Ins(1,3,4,5)P_4 may be more sensitive to subtle structural changes in PTEN than that using PtdIns(3,4,5)P_3.

Ability of PTEN to Bind to LMVs. During our study of PTEN phosphatase activities, a second biochemical property of PTEN was reported: the ability to bind to lipid membranes in vitro through the C2 domain (33). This raised the possibility that some PTEN mutations may inactivate the physiological function of PTEN through disruption...
of the membrane binding activity. To examine this possibility, 15 mutant PTENs with (S10N, L42R, K289E, D331G, V369G, and T401I) or without (Y16C, Y68H, R130Q, R130L, G165R, S170N, R173C, G251C, and L345Q) phosphatase activity were subjected to a protein-membrane binding assay using LMVs that consisted of three different phosphoglycerides (PC, PS, and PE) according to procedures published previously (31, 32). Representative data of the selected mutant PTENs are shown in Fig. 4. When bacterial proteins copurified with PTEN failed to bind LMVs, all mutants tested retained the binding activity of PTEN, irrespective of whether or not they had phosphoinositide phosphatase activity. This result suggested that the ability of PTEN to bind to membranes is not a major target of tumor-derived or germ-line-derived missense mutations, even if the activity may be important for physiological PTEN function.

Discussion

Since its discovery, PTEN has been thought to be a protein phosphatase because it shares the signature motif HCXXGXXR with the active site of PTPs and dual-specific protein phosphatases. However, recent studies have shown that the biologically relevant targets of PTEN may not be phosphoproteins but rather a subset of phosphoinositides (20, 22, 25). This speculation can be supported by several findings. For example, tumor cell lines with mutant PTEN retained elevated levels of PtdIns(3,4,5)P3 and Akt activity, and the introduction of wild-type PTEN reduced the levels of both (34, 35). Furthermore, analysis of the PTEN crystal structure indicated that the phosphatase active site of PTEN is larger than that of the PTPs and that the COOH-terminal portion has a structure similar to the C2 domain and actually binds to phospholipid membranes in vitro (33). To elucidate the most important tumor suppressor function of PTEN, the analysis of the effect of the missense mutations on currently known functional properties of PTEN will be beneficial until all of the functions of PTEN are clarified. Thus far, only a limited number of tumor-derived and germ-line-derived PTEN missense mutations have been examined for known PTEN functions. All of the missense mutations that have been tested inactivated phosphoinositide phosphatase activity, whereas some of the mutations retained protein phosphatase activity (22, 25). Although these observations stressed the importance of phosphoinositide phosphatase activity, it was necessary to determine whether the reported PTEN missense mutations inactivate the currently proposed functions of PTEN.

As an initial study, we constructed 42 missense mutations, which covered more than 40% of reported missense mutations, mapped throughout from the NH2-terminal and COOH-terminal portions of PTEN and tested them against the phosphoinositide phosphatase activity and membrane binding activity of wild-type PTEN. We classified them according to the recent report describing PTEN crystal structure (33). There were 31 mutations (S10N, Y16C, G20E, Y27S, L42R, H61R, Y68H, C71Y, H93Y, C105F, D107Y, L112P, L112R, A121P, C124R, G129R, G129E, R130G, R130L, R130Q, V133I, M134L, C136Y, Y155C, G165R, S170N, S170R, R173C, R173H, R173P, and Y174N) within the NH2-terminal phosphatase domain (residues 7–185), 9 mutations (S227F, G251C, K289E, D331G, F341V, K342N, V343E, L345Q, and F347L) within the COOH-terminal C2 domain (residues 186–351), and 2 mutations (V369G and T401I) close to the COOH-terminal end. Among these mutations, most of the mutations within the NH2-terminal phosphatase domain (27 of 31 mutations, 87%) inactivated phosphatase activity. In contrast, a higher percentage of mutations within both the C2 domain (five out of nine mutations, 56%) and the COOH-terminal end (two of two mutations, 100%) retained phosphatase activity. Overall, our results showed that most mutations (38 of 42 mutations, 90%) eliminated or reduced phosphatase activity. The mutations within the phosphatase domain contain eight mutations located at the predicted active site pocket, including six mutations (C124R, G129R, G129E, R130G, R130L, and R130Q) in the HCXXGXXR signature motif of the P loop (residues 123–130) at the bottom of the pocket and two mutations (H93Y and G165R) at the walls of the pocket, the WPD loop (residues 90–97), and the TI loop (residues 160–168), respectively. All mutations within the active site pocket eliminated phosphatase activity. The mutations in the motif represent 19% (27 of 143) of the total missense mutations according to our statistics. From our functional analysis and the previous genetic and structural analyses, we conclude that PTEN phosphoinositide phosphatase activity is an important tumor-suppressive function of PTEN.

However, 26% (11 of 42) of the mutants retained some level of the normal PTEN phosphatase activity in vitro. Although there may be a polymorphism(s) among these mutants, it is likely that the in vitro phosphatase assays do not always reflect the in vivo phosphatase activity of PTEN and that there might be other mechanisms of PTEN inactivation. In our study, most of the mutations that mapped to the COOH-terminal region retained some levels of phosphoinositide phosphatase activity. According to recent studies, there may be regulatory domains in the COOH-terminal region. Georgescu et al. (30) have shown that two missense mutations (L345Q and T348I) in the predicted β-strand structure (residues 342–349) seem to destabilize PTEN in mammalian cells, although these mutations are also defective in the phosphatase assay by bacterially expressed proteins. Lee et al. (33) have shown that the predicted C2 domain in the COOH-terminal portion bound to phospholipid membranes. They also speculated that the C2 and the phosphatase domains associate across an extensive interface adjacent to the phosphatase active site. Furthermore, they predicted that the C2 domain may not only recruit PTEN to the membrane but may also help with the positioning and orientation of the catalytic domain with respect to the membrane-bound substrate. In this study, we could not find any tumor-derived mutations that affected the membrane binding activity of PTEN, which suggested that the binding of PTEN to membranes is not a major target of the missense mutations. Our results do not exclude the possibility of binding activity as an important tumor suppressor function of PTEN because there are high frequencies of PTEN frameshift (39%) and nonsense (18%) mutations that are predicted to eliminate the PTEN membrane binding domain and perhaps preserve enzyme activity. It would be of great interest to determine whether the mutations in the C2 domain, which have both in vitro phosphatase and membrane binding activities, affect the phosphatase activity under the conditions of PTEN protein binding to the phospholipid membrane.

PTEN also has another domain, called the PDZ binding domain, in the COOH-terminal end (33, 36); the domain binds to the PDZ proteins (DLG1 and MATH205), and this binding is regulated by phosphorylation of the tyrosine residue at codon 401. It would also be
FUNCTIONAL EVALUATION OF PTEN MUTATION


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*Cancer Res* 2000;60:3147-3151.

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