Conformational Stability and Catalytic Activity of PTEN Variants Linked to Cancers and Autism Spectrum Disorders

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Supporting Information

ABSTRACT: Phosphoinositides are membrane components that play critical regulatory roles in mammalian cells. The enzyme PTEN, which catalyzes the dephosphorylation of the phosphoinositide PIP3, is damaged in most sporadic tumors. Mutations in the PTEN gene have also been linked to autism spectrum disorders and other forms of delayed development. Here, human PTEN is shown to be on the cusp of unfolding under physiological conditions. Variants of human PTEN linked to somatic cancers and disorders on the autism spectrum are shown to be impaired in their conformational stability, catalytic activity, or both. Those variants linked only to autism have activity higher than the activity of those linked to cancers. PTEN-L, which is a secreted trans-active isoform, has conformational stability greater than that of the wild-type enzyme. These data indicate that PTEN is a fragile enzyme cast in a crucial role in cellular metabolism and suggest that PTEN-L is a repository for a critical catalytic activity.

Phosphatase and tensin homologue on chromosome 10 (PTEN, EC 3.1.3.67) catalyzes the hydrolysis of phosphatidylinositol-3,4,5-trisphosphate (PIP3) to form phosphatidylinositol-4,5-bisphosphate (PIP2). Its PIP3 substrate activates the phosphatidylinositol-3-kinase (PI3K) Akt pathway that mediates cell proliferation. Hence, PTEN is a tumor suppressor whose recognized importance in human biology is increasing steadily. In addition to lipid phosphatase activity on the plasma membrane, which could alone contribute significantly to tumor suppression, PTEN has important roles in nuclear processes that include the promotion of chromosomal stability, repair of DNA damage, and regulation of the cell cycle.

The PTEN gene is mutated in 50–80% of sporadic human cancers. Moreover, germline mutations in PTEN are associated with the molecularly defined PTEN hamartoma tumor syndromes (PHTSs). PHTSs include Cowden syndrome and Bannayan–Riley–Ruvalcaba syndrome and are associated with increased cancer predisposition. PTEN mutations are also strongly associated with macrocephaly.

Some patients on the autism spectrum have germline mutations in the PTEN gene. Autism spectrum disorders (ASDs) are characterized by impaired social interactions, impaired verbal or nonverbal communication skills, and repetitive behaviors. ASDs are enigmatic: despite being heritable, defects in a single gene or set of genes do not seem to be present in all patients. The biochemical link between PTEN dysfunction and ASDs is not known, though loss of PTEN function in developing mouse brain cells leads to their overgrowth, and deletion of the PTEN gene in neurons has large effects on neuronal morphology and circuitry.

Recently, alternative translation of the PTEN mRNA using an upstream CTG codon was discovered and found to result in the production of a secreted trans-active version of PTEN. This isoform is able to shrink PTEN-null tumors in mice. The isoform has also been observed in the cytosol of cells and controls cell death upon interaction with mitochondria. Wild-type PTEN and PTEN-L have similar kinetic parameters for catalysis of PIP3 hydrolysis.

Conformational stability is critical to the biological function of enzymes and other proteins. Mutations that lead to unstable human proteins can be lead to disease. For example, p53, like PTEN, is a tumor suppressor protein that is altered in a large fraction of human cancers. Extensive analyses have demonstrated that many cancer-associated p53 variants have compromised thermostability. That compromise also afflicts angiogenin, a human ribonuclease that has neuroprotective activity but is damaged in some patients with amyotrophic lateral sclerosis. To date, however, no data about the thermostability of PTEN or its disease-related variants have been reported.

In this work, we present a biochemical analysis of variants of PTEN that are linked to somatic cancer, PHTSs, and ASDs. First, we analyze the catalytic activity of these variants using a newly developed continuous assay. Then, we develop an assay to measure the conformational stability of PTEN and its variants, including PTEN-L, under a variety of conditions. The

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ensuing data provide the necessary biochemical insight into the relationship between PTEN dysfunction and human disease.

**EXPERIMENTAL PROCEDURES**

**Materials.** *Escherichia coli* BL21(DE3) cells for protein production were from Novagen (Madison, WI). Expression plasmid pET30B-PTEN was a gift from A. Ross (plasmid 20741 from Addgene, Cambridge, MA) and directs the expression of human PTEN with a C-terminal His$_8$ tag.$^{17}$ Plasmids that direct the production of PTEN variants were created with the QuikChange site-directed mutagenesis kit from Agilent (Santa Clara, CA). Expression plasmid pET30B-PTEN-L-S was derived by Gibson assembly,$^{25}$ using a gBlocks gene fragment from Integrated DNA Technologies (Coralville, IA). In pET30B-PTEN-L-S, the CTG encoding residue 1 was replaced with an ATG, the signal sequence (codons 2–21) was removed, the ATG at the translation start site for wild-type PTEN was replaced with ATGA (codon 174), and other codon optimizations for *E. coli* expression that did not affect the protein sequence in codons 2–173 were implemented.$^{14,17}$

Terroric broth (TB) contained tryptm (12 g), yeast extract (24 g), K$_2$HPO$_4$ (72 mM), KH$_2$PO$_4$ (17 mM), and glycerol (4 mL). Columns of HisTrap HP, HiTrap Q HP, HiTrap Heparin HP, and Superdex G200 resins for protein purification were from GE BioSciences (Piscataway, NJ).

d$_{50}$,Phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$) was from Avanti Polar Lipids (Alabaster, AL) and Echelon Biosciences (Salt Lake City, UT). Bacterial nucleoside phosphorylase (PNPase) was product N2415 from Sigma-Aldrich (St. Louis, MO), dissolved in reaction buffer, and buffer-exchanged to remove residual phosphate. 7-Methyl-6-thioguanosine (MESG) was from Berry and Associates (Dexter, MI). Dithiobutylamine (DTBA)$^{23}$ was product 774405 from Agilent Technologies. Data were analyzed with Prism version 6 from Graphpad (San Diego, CA). The value of $T_m$ was the temperature at the midpoint of the thermal transition between the low fluorescence of folded protein and the high fluorescence of unfolded protein after fitting to the Boltzmann equation.$^{26}$

**Production and Purification of PTEN.** Methods for the expression and purification of PTEN were based on those of Ross and co-workers.$^{24}$ PTEN expression plasmids were transformed into *E. coli* strain BL21(DE3) and grown in TB supplemented with kanamycin (30 μM). Expression was induced at an OD of 0.5–0.6 at 600 nm by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.10 mM, and cells were grown for 20–22 h at 18 °C. Cells were harvested by centrifugation and lysed in 20 mM sodium phosphate buffer (pH 7.4) containing NaCl (0.50 M), imidazole (20 mM), and β-mercaptoethanol [0.7% (v/v)] with a French pressure cell. Lysate was clarified by centrifugation at 20000g, and the soluble fraction was applied to a HisTrap HP column. Protein was eluted with 0.50 M imidazole, and fractions containing PTEN were purified further by chromatography on a HiLoad 26/60 G200 Superdex gel filtration column. As a final step, wild-type PTEN and variants were purified by chromatography on a HiTrap Q anion-exchange column at pH 7.4, and PTEN-L was purified by chromatography on a HiTrap Heparin HP affinity column using an AKTA system from Amersham-Pharmacia (Piscataway, NJ), and the results were analyzed with the UNICORN Control System. Aliquots of protein were supplemented with dithiothreitol (10 mM), glycerol [25% (v/v)], and ethylendiaminetetraacetic acid (EDTA) (2 mM); flash-frozen in liquid nitrogen; and stored at −80 °C. Protein concentrations were measured with a Bradford assay.$^{25}$

**Assays of Conformational Stability.** The conformational stabilities of wild-type PTEN and its variants were assessed via differential scanning fluorimetry (DSF).$^{26}$ This technique relies on the increase in the fluorescence of the dye SYPRO Orange upon its binding to hydrophobic residues of a protein that are exposed during thermal denaturation. Wild-type PTEN or a variant was dissolved at a concentration of 0.1 μg/μL in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH buffer (pH 7.4) containing NaCl (100 mM) and DTBA (10 mM). SYPRO Orange, which is supplied at a concentration of 5000X, was added to a concentration of 15X. In experiments that aim to probe molecular crowding, Ficoll PM 70 or sucrose was present at a concentration of 50, 100, 150, 200, 250, or 300 mg/mL. Assay solutions were exposed to a temperature gradient from 10 to 95 °C with an increase of 1.0 °C/min. When the solutions were heated, their fluorescence was monitored by using excitation at (580 ± 10) nm and emission at (623 ± 14) nm. Heating and fluorescence monitoring were performed with a ViiA 7 Real-Time PCR System from Life Technologies. Assays were also performed in PBS rather than HEPES buffer, in the presence of calcium ions (added from 0.1 μM to 1.2 mM), or with a heating rate of 0.2 °C/min rather than 1.0 °C/min.

Thermal denaturation data were prepared for analysis with ViiA 7 version 1.0 and analyzed with Protein Thermal Shift version 1.2, both from Life Technologies. Subsequent data analysis and plotting were performed with Prism version 6 from Graphpad. The value of $T_m$ was the temperature at the midpoint of the thermal transition between the low fluorescence of folded protein and the high fluorescence of unfolded protein after fitting to the Boltzmann equation.$^{26}$

**Assays of Enzymatic Activity.** Assays of the catalytic activity of wild-type PTEN and its variants were performed by using the continuous assay described previously.$^{17}$ Briefly, the PNPase concentration was measured with a Bradford assay$^{25}$ and added to a concentration of 57 μg/mL (2 μM) in reaction buffer, which was 50 mM Tris-HCl buffer (pH 7.6) containing EDTA (2.0 mM), MESG (0.40–0.60 mM), and DTBA (40 mM). Known concentrations of PIP$_3$, substrate (0–320 μM) were added to the buffer, and reactions were initiated by the addition of PTEN. Wild-type PTEN was used at a concentration of 10 nM and PTEN-L at 20 nM, and PTEN variants were used at concentrations of 100–300 nM. Measurements of absorbance at 360 nm were recorded at 15 °C with a Cary 60 UV–vis spectrophotometer having a Varian Cary Single Cell Peltier temperature control accessory from Agilent Technologies. Data were analyzed with Prism version 6 from Graphpad.

**RESULTS**

**Thermostability of Wild-Type PTEN.** We attempted to measure the conformational stability of wild-type PTEN with several methods, including thermal denaturation or chemical denaturation as monitored by ultraviolet spectroscopy or circular dichroism spectroscopy. In our hands, only DSF$^{26}$ provided a precise measure of conformational stability (Figure
Figure 1. Ribbon diagram of the three-dimensional structure of human PTEN. The enzyme has a phosphatase domain (green) and C2 domain (gray). The side chains of amino acid residues that are substituted in cancers (red) and autism spectrum disorders (blue) are shown explicitly. The enzymic active site contains an L-(-)-tartrate ion, which is rendered in ball-and-stick format. The structure lacks 7 and 49 residues at the N- and C-termini, respectively, and a 24-residue internal loop. The image was created with PyMOL from Schrödinger (New York, NY) and Protein Data Bank entry 1d5r.37

(Table 1). This value was not affected by the presence of calcium ions at intracellular (0.1–0.4 μM) or extracellular (1.2 mM) concentrations (data not shown).

Table 1. Parameters of Wild-Type PTEN and Dysfunctional Variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>$T_m$ °C ± s</th>
<th>$k_{cat}/K_m$ (μM/$\text{min}^{-1}$) ± s</th>
<th>$k_{cat}$ (min$^{-1}$) ± s</th>
<th>$K_m$ (μM) ± s</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type PTEN</td>
<td>40.3 ± 0.1</td>
<td>174 ± 40</td>
<td>4000 ± 300</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>C124S</td>
<td>40.8 ± 0.1</td>
<td>&lt;0.03</td>
<td>ND$^c$</td>
<td>ND$^c$</td>
</tr>
<tr>
<td>N82T</td>
<td>39.2 ± 0.3</td>
<td>27 ± 6</td>
<td>1300 ± 200</td>
<td>48 ± 18</td>
</tr>
<tr>
<td>F37YS</td>
<td>32.2 ± 0.2</td>
<td>16 ± 5</td>
<td>700 ± 140</td>
<td>45 ± 21</td>
</tr>
<tr>
<td>H91R</td>
<td>38.7 ± 0.3</td>
<td>4.5 ± 0.4</td>
<td>171 ± 9</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>Y176C</td>
<td>39.0 ± 0.4</td>
<td>55 ± 10</td>
<td>1800 ± 180</td>
<td>32 ± 9</td>
</tr>
<tr>
<td>E157G</td>
<td>38.4 ± 0.5</td>
<td>49 ± 11</td>
<td>1400 ± 150</td>
<td>28 ± 9</td>
</tr>
<tr>
<td>PTEN-L</td>
<td>46.7 ± 0.2</td>
<td>100 ± 25</td>
<td>477 ± 28</td>
<td>48 ± 1.1</td>
</tr>
</tbody>
</table>

$^a$Values (±standard error) were determined by DSF in 100 mM HEPES-NaOH buffer (pH 7.4) containing NaCl (100 mM), DTBA (10 mM), and SYPRO Orange Protein Gel Stain (15×) with heating at a rate of 1 °C/min. Data are shown in Figure 2 and Figure S1 of the Supporting Information. $^b$Values (±standard error) are for the turnover of diC$_2$-phosphatidylinositol-3,4,5-trisphosphate in 50 mM Tris-HCl buffer (pH 7.6) containing NaCl (0, 100, or 200 mM), EDTA (2.0 mM), MESG (0.20 mM), and DTBA (40 mM), as initiated by the addition of PTEN to a final concentration of 20 nM. Values (±standard error) were derived by fitting initial velocity data to the Michaelis–Menten equation. $^c$Not determined. $^d$Values from ref 17.

Thermostability of PTEN-L. We assessed the conformational stability of PTEN-L, the “long” isoform of PTEN, with DSF. Strikingly, we found that PTEN-L, which has 173 additional residues on its N-terminus, has a $T_m$ of (46.7 ± 0.2) °C (Figure 2). This value is 6.4 °C greater than that of wild-type PTEN.

Effect of Inorganic Phosphate and Heating Rate on the Value of $T_m$. Ligands have long been known to increase the conformational stability of proteins$^{27–29}$ along with their resistance to proteolysis.30 As PTEN is a phosphatase, we assessed its thermostability in PBS, a buffer that is isotonic with human cells and contains 11.8 mM inorganic phosphate. We found that the $T_m$ value of wild-type PTEN is (42.9 ± 0.1) °C in PBS (Figure 2), an increase of 2.6 °C compared to that in HEPES buffer. In contrast, the $T_m$ value of PTEN-L is (46.2 ± 0.1) °C in PBS, which is actually a slight decrease compared to that in HEPES buffer. These data suggest that wild-type PTEN has an affinity for inorganic phosphate greater than that of PTEN-L.

Like the binding of ligands, the rate of sample heating is also known to affect the measured value of $T_m$.31 Slower rates allow the folded and unfolded states of a protein to equilibrate more extensively and can give rise to lower $T_m$ values. Instead of the typical heating rate of 1 °C/min for a DSF experiment,32 we used a rate of 0.2 °C/min. We found that the measured $T_m$ values for wild-type PTEN and PTEN-L in HEPES buffer decrease to (35.6 ± 0.1) °C and (41.6 ± 0.2) °C, respectively, with the slower heating rate (Figure 2). Likewise, the $T_m$ values in PBS decrease upon slow heating to (39.0 ± 0.1) and (42.4 ± 0.2) °C, respectively. Again, PTEN-L is much less affected by inorganic phosphate than is wild-type PTEN. Indeed, the Δ$T_m$ of 6 °C in HEPES buffer and the Δ$T_m$ of 3 °C in PBS are independent of heating rate. We performed all subsequent measurements of thermostability in the absence of inorganic phosphate to prevent confounding ligand binding with conformational stability.

Effect of Molecular Crowding on the Value of $T_m$. The concentration of molecules in the cytosol approaches 300 mg/mL,32 and is even greater in the nucleus.33 The conformational stability of proteins can be increased by the ensuing molecular crowding.32,34,35 To determine whether molecular crowding affects the conformational stability of PTEN, we determined the value of $T_m$ for wild-type PTEN in the presence of a molecular crowding agent, Ficoll PM 70 (0–300 mg/mL), which is a high-molecular weight, highly branched polymer of sucrose. We found that the $T_m$ value increases with the concentration of Ficoll PM 70 (Figure 3). At 300 mg/mL Ficoll PM 70, which mimics the concentration of macromolecules in the cytosol,32 the $T_m$ value is increased by 2 °C. To control for...
specific interactions with the sucrose units, we also determined the value of $T_m$ for wild-type PTEN in the presence of sucrose itself (0–300 mg/mL). We found that the $T_m$ value has an even greater dependence on the concentration of sucrose than on that of Ficoll PM 70. At 300 mg/mL sucrose, the $T_m$ value of PTEN is increased by 4 °C.

**Cancer-Linked Variants of PTEN.** We produced and purified three variants of PTEN that have been found in somatic tumors as well as in the germline of patients with PHTSs (Figure 1). We began with an active-site variant. Catalysis by PTEN entails nucleophilic attack by the sulfur of PHTSs (Figure 1). We began with an active-site variant.

**Dismutative catalysis.** Indeed three variants of PTEN that have been found in somatic tumors as well as in the germline of patients with PHTSs (Figure 1). We found that H93R PTEN had a low $T_m$ value of (39.2 ± 0.4) °C, which is close to physiological temperature in humans.

**Discussion.** PTEN has a fragile conformation. Human PTEN has an elongated structure composed of two distinct domains (Figure 1). The conformational stability of this structure has not been assessed previously. The raw data from thermal denaturation experiments were indicative of a transition between two states, not among three (Figure S1 of the Supporting Information). Apparently, the two domains of PTEN unfold cooperatively rather than independently. This single unfolding transition gave rise to a $T_m$ value that is close to the physiological temperature of humans (Figure 2).

We found that the $T_m$ value of PTEN correlates with the concentration of Ficoll PM 70 (Figure 3), a sucrose polymer that can mimic molecular crowding in cellulo.33 We also found, however, that much of this added stability arises from specific interactions between the sucrose units and PTEN, as a sucrose molecule confers approximately twice the increase in the $T_m$ value as does a sucrose unit within the Ficoll PM 70 polymer. We conclude that molecular crowding does not lead to a substantial increase in the thermostability of PTEN. Hence, PTEN is poised on the edge of instability, and a point mutation that leads to a small decrease in PTEN thermostability can have detrimental consequences in cellulo.

The fragility of PTEN is in marked contrast to that of another important tumor suppressor, p53. Recently, Veprintsev,
Fersht, and co-workers used DSF to show that human p53 has a $T_m$ value of 46 °C,\textsuperscript{20} which is well above that of human PTEN under any condition used herein (Figures 2–4). These data suggest that PTEN is more vulnerable than p53 to inactivation in cellulo by an amino acid substitution.

![Figure 4. Thermostability of human PTEN and its variants. Values of $T_m$ were determined with DSF ($\Delta T = 1{^\circ}C/min$) in 100 mM HEPES-NaOH buffer (pH 7.4) containing NaCl (100 mM), DTBA (10 mM), and SYPRO Orange Protein Gel Stain (15X). Each data point is for an individual experiment. Data for wild-type PTEN and PTEN-L are from Figure 2. Raw data are shown in Figure S1 of the Supporting Information. Values of $T_m$ are listed in Table 1.](image)

Unlike human PTEN, human PTEN-L is not a fragile protein. Its $T_m$ value is 10 °C above physiological temperature. Previously, the 173 appended residues were shown to allow secretion of PTEN-L and uptake by other cells.\textsuperscript{14} Our data indicate that these residues also serve to endow PTEN with greater conformational stability. That attribute befits a protein that must survive in the extracellular matrix and provides humans with a reservoir of PTEN activity that is less vulnerable to inactivation.

The three-dimensional structure of PTEN-L is unknown. Like wild-type PTEN, PTEN-L unfolds with a single transition (Figure S1 of the Supporting Information). This cooperative unfolding, along with the conformational stability conferred by the appended residues, is consistent with the 173 N-terminal residues of PTEN-L adopting a defined three-dimensional structure that interacts intimately with one or both of its globular domains. Kinetic data suggest that the N-terminal residues of PTEN-L interact with the PIP$_2$-binding motif (residues 6–14 of wild-type PTEN).\textsuperscript{17} These experimental data conflict with a bioinformatic analysis predictive of a disordered N-terminus in PTEN-L.\textsuperscript{41}

**Cancer-Linked Variants of PTEN.** The importance of PTEN to cancer progression and many other cellular functions and malfunctions cannot be understated.\textsuperscript{3} We characterized three variants of PTEN that are linked to cancers. We found that two of these three variants are deficient in either conformational stability or catalytic activity. C124S PTEN lacked detectable catalytic activity; F337S PTEN has a $T_m$ that is nearly 10 °C lower than that of wild-type PTEN and would be unfolded in cellulo. In contrast, N82T PTEN is less compromised than the other two variants. This finding is consistent with the in vivo data of Newschaffer and co-workers, who reconstituted a humanized PI3K/PTEN system in *Saccharomyces cerevisiae* cells.\textsuperscript{42} The N82T substitution is linked with early onset breast cancer. We note that Asn82 is located on the surface of the protein and could play a role in PTEN–protein interactions.\textsuperscript{43} Moreover, the installation of a threonine residue at position 82 converts this segment of the protein into a putative substrate for the cellular kinases STE, STE7, and MAP2K2.\textsuperscript{44} Phosphorylation there could be detrimental to PTEN activity in cellulo.

### Autism-Linked Variants of PTEN

All three of the variants of PTEN tested herein exhibited $T_m$ values between 38 and 39 °C. Two of these variants (E157G and Y176C) were still efficacious catalysts with $k_{cat}/K_M$ values near 50 μM$^{-1}$ s$^{-1}$, which is \(1/3\) of that of the wild-type enzyme. These data are again consistent with observations in *S. cerevisiae* cells.\textsuperscript{42} Another variant (H93R) is found in patients with PHTS as well as in patients with somatic cancer.\textsuperscript{36,45} This variant had been analyzed previously\textsuperscript{39} and found to be defective in binding to the product PIP$_3$, an event that is important for the manifestation of the catalytic activity of PTEN.\textsuperscript{37} We too found that the H93R PTEN has low catalytic activity.

### CONCLUSIONS

We have reported the first measurements of the thermostability of human PTEN. We find that PTEN is a quasi-stable protein at physiological temperature, even in the presence of a ligand (inorganic phosphate) or a molecular crowding agent. PTEN dysfunction leads to the overgrowth, excessive proliferation, and accelerated differentiation of human cells.\textsuperscript{3,12} Accordingly, we have also reported a biochemical analysis of six PTEN variants that are implicated in somatic cancers and diseases on the autism spectrum. We find that those variants linked to cancers are more damaged than those linked to autism. This conclusion, which is based on biochemical data (Table 1), is consistent with observed effects of allelic mutations in cellulo.\textsuperscript{46} Notably, the PTEN-L isoform is more stable than wild-type PTEN. As both PTEN and PTEN-L are encoded by the same gene, the uptake of PTEN-L could provide a cell with some relief from a deleterious mutation. In addition to underpinning an understanding of the biology and pharmacology of PTEN, these findings encourage the development of small-molecule ligands that stabilize disease-related variants of PTEN and thus enhance their enzymatic activity in cellulo.

### ASSOCIATED CONTENT

**Supporting Information**

DSF raw data (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

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