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PTEN catalysis of phospholipid dephosphorylation reaction follows a two-step mechanism in which the conserved aspartate-92 does not function as the general acid — Mechanistic analysis of a familial Cowden disease-associated PTEN mutation

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Abstract

PTEN exerts its tumour suppressor function by dephosphorylating the phospholipid second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). Herein, we demonstrate that the PTEN-catalysed PIP_3 dephosphorylation reaction involves two-steps: (i) formation of a phosphoenzyme intermediate (PE) in which Cys-124 in the active site is thiophosphorylated, and (ii) hydrolysis of PE. For protein tyrosine- and dual-specificity phosphatases, catalysis requires the participation of a conserved active site aspartate as the general acid in Step 1. Its mutation to alanine severely limits PE formation. However, mutation of the homologous Asp-92 in PTEN does not significantly limit PE formation, indicating that Asp-92 does not act as the general acid. G129E is a common germline PTEN mutations found in Cowden syndrome patients. Mechanistic analysis reveals that this mutation inactivates PTEN by both significantly slowing down Step 1 and abolishing the ability to catalyse Step 2. Taken together, our results highlight the mechanistic similarities and differences between PTEN and the conventional protein phosphatases and reveal how a disease-associated mutation inactivates PTEN.

Keywords: PTEN; Tumour suppressor; Catalytic mechanism; Phospholipid; Second messenger; Phosphatase; Cancer; Neuronal death; Cowden disease

1. Introduction

The tumour suppressor PTEN is a novel phosphatase displaying both phosphoprotein- and phospholipid phosphatase activities. It shares homology within its active site with the protein tyrosine phosphatases (PTPs) and the dual-specificity protein phosphatases (DSPs) (see [1] for review). The physiological phospholipid substrate of PTEN is phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3), a phospholipid second messenger involved in the regulation of cell growth, proliferation and cell death [2]. The tumour suppressor function of PTEN is absolutely dependent upon its phospholipid phosphatase activity [3] while both the phospholipid and phosphoprotein phosphatase activities are involved in the signaling pathway governing neuronal cell death [4].

Somatic mutations of PTEN contribute to the development of many types of human cancer including glioblastoma, malenoma, prostate, breast, lung, and endometrial cancer (see [5] for review). For breast cancer cells overexpressing the oncogenic receptor tyrosine kinase ErbB2, binding of trastuzumab, a humanized monoclonal antibody against the extracellular domain of ErbB2 can suppress breast cancer growth. The study by Nagata et al. indicates that PTEN is an obligatory component mediating to tumour suppressor action of

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trastuzumab [6]. In addition to the somatic mutations that contribute to a wide spectrum of human cancer, germline mutations of PTEN gene are frequently found in patients suffering from Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome [5]. Cowden syndrome patients develop a kind of mucocutaneous lesions called hamartomas and have a much higher risk of developing carcinoma of breast and thyroid gland [5]. The G129E mutation is a PTEN active site germline mutation frequently associated with Cowden syndrome. Since the resultant PTEN mutant lacks phospholipid phosphatase activity and yet retains protein phosphatase activity, it have been used by many researchers as a tool to define the roles of the phospholipid phosphatase activity of PTEN in tumour suppression [7,8]. How the G129E mutation abolishes the phospholipid phosphatase activity was not known.

The active site of PTEN contains the phosphate-binding loop (P-loop) of the consensus sequence HCXXGXXR and the "Trp-Pro-Asp"-loop (also called the WPD loop) commonly found in PTPs and DSPs [9]. Mutations of several conserved residues in the active site of PTEN, including Cys-124, Gly-129 and Arg-130 in the P-loop, as well as Asp-92 in the WPD loop, result in either a significant decrease or complete loss of catalytic activity [3,10–12]. Furthermore, the crystal structure of an active PTEN mutant reveals that the three-dimensional structure of the PTEN phosphatase domain is very similar to that of the phosphatase domain of PTPs and DSPs [11]. These data strongly suggest that, similar to PTPs and DSPs, PTEN catalysis follows a two-step mechanism that involves the formation of a covalent phosphoenzyme intermediate (PE) in Step 1 and hydrolysis of PE in Step 2 (see [1,13,14] for review). Experimental evidence confirming this notion has yet to be documented. The most definitive evidence is demonstration of the formation PE during the course of PTEN dephosphorylation of PIP_3. In this report, we provide the evidence of the existence of such an intermediate.

The dephosphorylation reaction catalysed by almost all known PTPs and DSPs requires the participation of a conserved Asp in the WPD loop [13,14]. In PTPs and DSPs, this Asp acts as the general acid in Step 1 by donating a proton to the bridging oxygen of the phosphoamino acid in the substrate. In Step 2, the deprotonated Asp acts as the general base, which polarizes the nucleophilic H_2O by accepting a proton from it, and orientates it to hydrolyse the PE. The PTEN crystal structure reveals that Asp-92, present in the Phe-Glu-Asp motif, corresponds to the conserved WPD loop Asp residue of PTPs and DSPs [11]. Intriguingly, our results reveal that, unlike the role played by the conserved Asp in the WPD loop of PTPs and DSPs, Asp-92 does not function as the general acid in Step 1 of the phospholipid dephosphorylation reaction, inferring that another functional group in the active site must fulfill the role as the general acid in the PTEN-catalysed dephosphorylation reaction.

Using the procedures we develop to trap PE and to measure the rate of hydrolysis of PE, we have defined the mechanistic basis by which Cowden disease-associated G129E mutation abolishes the PTEN phospholipid phosphatase activity. In light of the significance of the two PTEN phosphatase activities in cancer formation and neuronal apoptosis, specific inhibitors targeting each of the two PTEN catalytic activities are valuable tools for deciphering the cellular functions of PTEN. Results of our investigation may contribute to the development of such inhibitors.

2. Materials and methods

2.1. Generation of PTEN cDNA by RT-PCR and cloning of wild-type PTEN cDNA into the pGEX-6P-3 vector

Total cellular RNA was extracted from Y3 Rat myeloma cells using a RNA preparation kit from Life Technology. The coding region of PTEN was amplified by RT-PCR reaction using the Promega Access RT-PCR system. The primers used in the RT-PCR reactions are primer 1 (acg *cgg atc* cat gac agc cat cat caa aga g) and primer 2 (acc gcg tcg ac*c tcg ag*t cag act ttt gta att tgt gta tg). *BamH1* and *Xho1* sites were introduced in primer 1 and primer 2, respectively (restriction sites are underlined). The resultant full length RT-PCR product was cloned into the *pGEX-6p-3* vector (Amersham Biosciences) at *BamH1* and *Xho1* sites. The plasmid generated was called *pGEX-6P3-PTEN*. Since *GST-PTEN* encoded by the *pGEX-6P3-PTEN* rapidly lost its phosphatase activity during purification and storage (manuscript in preparation), we therefore expressed the recombinant PTEN and its mutants in baculovirus-infected Sf9 cells.

2.2. Construction of the baculovirus transfer plasmids for the expression of wild-type PTEN, [D92A]PTEN, [C124A]PTEN and [G129E]PTEN

Using the *pGEX-6P3-PTEN* plasmid as the template, full length PTEN cDNA was copied and amplified using primer 3 (acg c*gg atc c*ga tga cag cca tca tca aag ag) as the forward primer and primer 4 (gac t*ga att c*tc aga ctt ttg taa ttt gtg aat g) as the reverse primer. *BamH1* and *EcoR1* sites were introduced in primer 3 and primer 4, respectively (restriction sites are underlined). The PCR reaction was carried out in a volume of 100 μl using Pfu (Stratagene) as the DNA polymerase and following the conditions specified by the manufacturer. The resultant full length PCR product was digested with *BamH1* and *EcoR1*, purified and ligated directly into *pBacpak9* transfer vector (Clontech) predigested with *BamH1* and *EcoR1*. The resultant plasmid, called *pBacPAK9-PTEN*, was subsequently used to produce the recombinant PTEN baculovirus. For the construction of the *pBacPAK9-[D92A] PTEN*, *pBacPAK9-[G129E] PTEN* and *pBacPAK9-[C124A] PTEN* plasmids, *pBacPAK9-PTEN* was used as the template and the point mutations were introduced by site-directed mutagenesis as described previously [15].

2.3. Generation of the recombinant baculoviruses for the expression of PTEN and PTEN mutants in Sf9 insect cells

The *pBacPAK9-PTEN* and the *pBacPAK9* plasmids of PTEN mutants were each co-transfected with *Bsu36 I*-digested *BacPAK6* viral DNA into Sf9 cells following procedures recommended by the manufacturer (Clontech). Individual recombinant viruses generated from the transfected Sf9 cells were purified by plaque assay. The presence of the recombinant PTEN and its mutants in the infected Sf9 cells was monitored by Western blotting using a polyclonal anti-PTEN antibody we generated as described in our previous report [16].

2.4. Purification of the recombinant wild PTEN, [D92A]PTEN, [G129E]PTEN, and [C124A]PTEN from baculovirus-infected Sf9 cells

A large-scale culture (1 l, $0.6–0.8 \times 10^6$ cells per ml) of Sf9 cells was infected with the recombinant baculovirus to direct the overexpression of PTEN and its mutants. The infected cells were harvested 50 h after infection for protein purification. All of the extraction and purification procedures were carried out at 4 °C. Cells were pelleted at 1000 ×*g* for 10 min, washed with Grace's serum-free medium, and homogenized in *Extraction Buffer* (25 mM Hepes, pH 7, 1 mM EDTA, 0.15 mg/ml DTT, 0.2 mg/ml Benzamidine, 0.1 mg/ml PMSF, 0.1 mg/ml Soybean Trypsin Inhibitor). The homogenate was clarified by centrifugation at 100,000 ×*g* for 40 min.

Recombinant PTEN or its mutants in the homogenate were purified by sequential chromatography on (i) a DEAE-Sepharose anion exchange column