Methods xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Yeast-based methods to assess PTEN phosphoinositide phosphatase activity *in vivo*

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ARTICLE INFO

Article history:
Received 15 September 2014
Received in revised form 16 October 2014
Accepted 17 October 2014
Available online xxxx

Keywords:
PTEN
Lipid phosphatase
Saccharomyces cerevisiae
Humanized yeast

ABSTRACT

The PTEN phosphoinositide 3-phosphatase is a tumor suppressor commonly targeted by pathologic missense mutations. Subject to multiple complex layers of regulation, its capital role in cancer relies on its counteracting function of class I phosphoinositide 3-kinase (PI3K), a key feature in oncogenic signaling pathways. Precise assessment of the involvement of PTEN mutations described in the clinics in loss of catalytic activity requires either tedious *in vitro* phosphatase assays or *in vivo* experiments involving transfection into mammalian cell lines. Taking advantage of the versatility of the model organism *Saccharomyces cerevisiae*, we have developed different functional assays by reconstitution of the mammalian PI3K–PTEN switch in this lower eukaryote. This methodology is based on the fact that regulated PI3K expression in yeast cells causes conversion of PtdIns(4,5)P_2 in PtdIns(3,4,5)P_3 and co-expression of PTEN counteracts this effect. This can be traced by monitoring growth, given that PtdIns(4,5)P_2 pools are essential for the yeast cell, or by using fluorescent reporters amenable for microscopy or flow cytometry. Here we describe the methodology and review its application to evaluate the functionality of PTEN mutations. We show that the technique is amenable to both directed and systematic structure–function relationship studies, and present an example of its use for the study of the recently discovered PTEN-L variant.

1. Introduction

PTEN (Phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor gene mapping at chromosome 10q23.3. It appears mutated in a plethora of both germ-line hereditary oncological syndromes and somatically developed tumors, including glioblastoma, prostate and endometrial carcinomas. The COSMIC database (Catalog Of Somatic Mutations In Cancer; cancer.sanger.ac.uk) currently compiles more than 3000 entries of diverse genetic PTEN variants from different tissue samples, 1174 of them (38%) corresponding to missense substitutions. Besides, PTEN has also been related to neurological disorders such as autism [1]. *PTEN* encodes a 403-amino acid phosphatase that can use both lipid and protein substrates. Recently, an alternative secreted 576-amino acid translational variant, named PTEN-L (also formerly known as PTEN-long) [2,3], has been described. The PTEN peptide folds into two major tertiary structure modules bearing respectively the phosphatase domain and a C2 membrane-binding domain. An N-terminal PtdIns(4,5)P_2-binding site overlapping a nuclear localization signal and a C-terminal tail containing PEST sequences and a PDZ-interaction signature are also features of PTEN [4]. In addition, the extra N-terminal extension of PTEN-L contains a secretion signal and sequences involved in its internalization into target cells [2].

The distinctive function of PTEN is the dephosphorylation of PtdIns(3,4,5)P_3, thus directly antagonizing class I PI3K and its downstream pathways, which involve protein kinases such as

Abbreviations: CWI, cell wall integrity; Gal, galactose; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide-dependent kinase; PH, pleckstrin homology; PI3K, phosphoinositide 3-kinase; PtdIns(4,5)P_2, phosphatidylinositol (4,5)-*bis*phosphate; PtdIns(3,4,5)P_2, phosphatidylinositol (3,4,5)-*tris*phosphate; *PTEN*, phosphatase and tensin homolog deleted on chromosome 10.

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http://dx.doi.org/10.1016/j.ymeth.2014.10.020
1046-2023/

PDK1, Akt and mTOR. This signaling hub governs multiple events promoting cell growth, proliferation and survival in most cell types, as well as other functions in specific tissues. Loss of PTEN function, in consequence, leads to sustained activation of PI3K-dependent signaling pathways. However, a large body of research along the last decade has revealed that PTEN plays both phosphatase-dependent and -independent roles [5,6]. Notably, a pool of nuclear PTEN is involved in repair of DNA damage [7,8]. In addition, PTEN is subject to multiple post-translational modifications that affect its conformation, activity and ability to interact with lipids or with other protein partners (rev in [6]), even with itself to form homodimers [9]. All these modifications, that include complex layers of phosphorylation, ubiquitylation, SUMOylation and acetylation, account for its finely tuned regulation. It is thus important to understand how particular PTEN mutations, either detected in patients or artificially generated to address the involvement of particular motifs in PTEN function and regulation, specifically affect its catalytic activity. Here, we describe heterologous expression in the yeast *Saccharomyces cerevisiae* as a platform that readily reproduces PTEN catalytic activity towards PtdIns(3,4,5)P_3 inside a living cell. Its exploitation for systematic analysis of PTEN mutations, especially missense substitutions leading to loss of function, is reviewed.

2. A yeast bioassay to assess the lipid phosphatase catalytic competence of PTEN mutations

Heterologous expression of the p110α catalytic subunit of mammalian PI3K in *S. cerevisiae* results in growth inhibition, as a consequence of conversion of essential PtdIns(4,5)P_2 pools into futile PtdIns(3,4,5)P_3 [10,11]. In order to disclose suitable experimental conditions to carry out studies on the PI3K pathway in the heterologous yeast model, we generated a series of constructs on the YCpLG vector (a centromeric *LEU2*-based vector based on YCplac111 provided with the *GAL1* promoter, a gift of J. Thorner, University of California at Berkeley, USA) that allowed galactose (Gal)-inducible expression of different versions of p110α. Namely, we expressed wild type p110α, an oncogenic H1047R mutation, C-terminal prenylatable p110α-CAAX, and N-terminal myristoylatable myr-p110α ([11,12] and unpublished data), yielding different degrees of toxicity in the yeast cell. Among them, we found that *GAL1*-driven induction of p110α-CAAX, but not a kinase-dead mutant version (p110α^{K802R}-CAAX), caused the most severe inhibition of yeast growth on solid medium. This 'humanized yeast system' has been exploited for testing candidate drugs [13] and performing drug screening in search for PI3K inhibitors [14]. Remarkably, co-expression of human PTEN-coding cDNA from the same *GAL1* promoter on the *URA3*-based pYES2 expression plasmid (Invitrogene™) fully suppressed PI3K-induced growth inhibition (Fig. 1). Furthermore, this was dependent on PTEN catalytic activity, because the canonical catalytically inactive C124S mutation was unable to restore growth (Fig. 2; [11]).

Based on these initial observations, we have thoroughly exploited this setting for the functional study of PTEN mutations. Considering that *S. cerevisiae* lacks both endogenous class I PI3K and PtdIns(3,4,5)P_3-dependent signaling [15], it is not to be expected that regulatory and functional aspects of PTEN other than its ability to counteract the toxic effects of co-expressed PI3K can be traced in this system. Thus, implementation of this alternative *in vivo* approach can contribute to discern how particular mutations specifically impinge on lipid phosphatase activity in an experimental setting deprived of the multiple layers of regulation (namely protein–protein interactions and post-translational modifications) that are present in mammalian cells. This said, caution must be taken when interpreting functional assays in this model as we ignore whether conserved post-translational protein modifications, like ubiquitylation or phosphorylation, may be acting on heterologous PTEN when expressed in yeast.

The system was applied to randomly screen for loss-of-function PTEN mutations by coupling error-prone PCR to *in vivo* gap-repair and negative selection, which often led to the isolation of missense mutations already isolated in the clinics [12]. Importantly, it can be readily used to test mutations found by clinicians and transferred to the yeast expression system by site-directed mutagenesis (see Section 2.1.3) [16]

2.1. Methods for yeast-based PTEN growth rescue assays

The methodology will be familiar to those trained in yeast genetics, involving co-transformation of YCpLG-p110α-CAAX and pYES2-PTEN, and subsequently monitoring growth assay on SC-Gal Ura- Leu- plates.

2.1.1. Yeast transformation

Any standard *S. cerevisiae* laboratory strain with an *ura3 leu2* genotype should be suitable for the assay. We routinely use the YPH499 strain (*MATa ade2-101 trp1-63 leu2-1 ura3-52 his3-Δ200 lys2-801*). Transformation of fresh competent cells can be achieved by the lithium acetate method [17]. Briefly, yeast cells freshly grown in 10 ml YPD medium (1% yeast extract, 2% peptone and 2% glucose) to an $OD_{600\ nm}$ of 0.5–1 are collected by centrifugation (1 min, 3000 rpm), transferred to an Eppendorf tube and re-centrifuged to carefully remove all remaining medium. The pellet is resuspended in 0.1 ml of sterile PEG4000 (40% v/v), 0.2 M lithium acetate, 1 μg/μl 2-mercaptoethanol. Alternatively, loaded loops of fresh yeast cells grown overnight on YPD agar can be resuspended in this solution until a thick suspension is achieved. Then 1 μl DNA of each plasmid (YCpLG-p110α-CAAX and pYES2-PTEN), miniprep

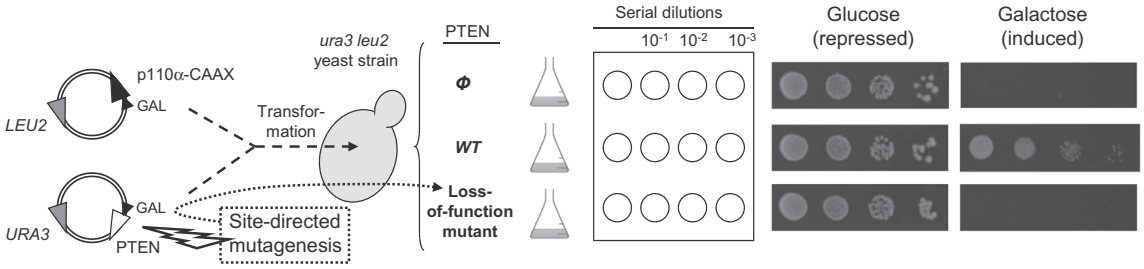

Fig. 1. Rationale for a yeast growth assay for PTEN phosphatase based on PTEN ability to rescue PI3K-induced growth inhibition. Co-expression of active PTEN with a prenylated version of p110α (p110α-CAAX), both under the *GAL1* regulatable promoter, relieves toxicity of the latter by restoring plasma membrane pools of PtdIns(4,5)P2, essential for the yeast cell [11]. Loss of this property can be detected on any mutant version of PTEN assayed. Collections of PTEN mutations can be generated by site-directed mutagenesis and assayed for their phosphatase activity by means of a simple growth vs. non-growth readout by using a serial dilution drop assay (method described in Section 2.1.2).

Please cite this article in press as: I. Rodríguez-Escudero et al., Methods (2014), http://dx.doi.org/10.1016/j.ymeth.2014.10.020