

Substrate specificity and acute regulation of the tumour suppressor phosphatase, PTEN

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Abstract

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a tumour suppressor that functions as a PtdIns(3,4,5) P_3 3-phosphatase to inhibit cell proliferation, survival and growth by antagonizing PI3K (phosphoinositide 3-kinase)-dependent signalling. Recent work has begun to focus attention on potential biological functions of the protein phosphatase activity of PTEN and on the possibility that some of its functions are phosphatase-independent. We discuss here the structural and regulatory mechanisms that account for the remarkable specificity of PTEN with respect to its PtdIns substrates and how it avoids the soluble headgroups of PtdIns that occur commonly in cells. Secondly we discuss the concept of PTEN as a constitutively active enzyme that is subject to negative regulation both physiologically and pathologically. Thirdly, we review the evidence that PTEN functions as a dual specificity phosphatase with discrete lipid and protein substrates. Lastly we present a current model of how PTEN may participate in the control of cell migration.

Introduction

PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a dual specificity, inositol lipid and protein tyrosine phosphatase that is mutationally inactivated or whose expression is lost in a wide range of sporadic human tumours. Germline mutations in PTEN are also responsible for human cancer predisposition syndromes such as Cowden's disease and Bannayan Riley Ruvalcaba syndrome and heterozygous PTEN^{+/-} mice often eventually die from

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multiple sporadic tumours [1–3]. Many of these effects can be attributed to the lipid phosphatase activity of PTEN through which it functionally antagonizes PI3K (phosphoinositide 3-kinase)-dependent signalling [2,4,5]. These observations have sparked enormous interest in understanding all aspects of PTEN function that contribute to understanding its pervasive impact in human disease. This article explores the biochemical characteristics of PTEN and how these relate to physiological functions. Firstly what is the range of inositide substrates utilized by PTEN *in vitro* and how might this differ in the cellular environment. Secondly, what is the significance of PTEN's protein tyrosine phosphatase activity and its relationship to tumour suppression. Thirdly, is the phosphatase activity dispensable for any functions attributable to the PTEN protein? One emerging concept that is being explored in our laboratory is the idea that PTEN exists in cells in a form that is constitutively active to a significant degree. PTEN therefore has a key role in maintaining PI3K-dependent lipid signals below the threshold required to initiate signalling. This would explain why loss of PTEN causes basal lipid signals to increase, leading to constitutive activation of the PI3K pathway.

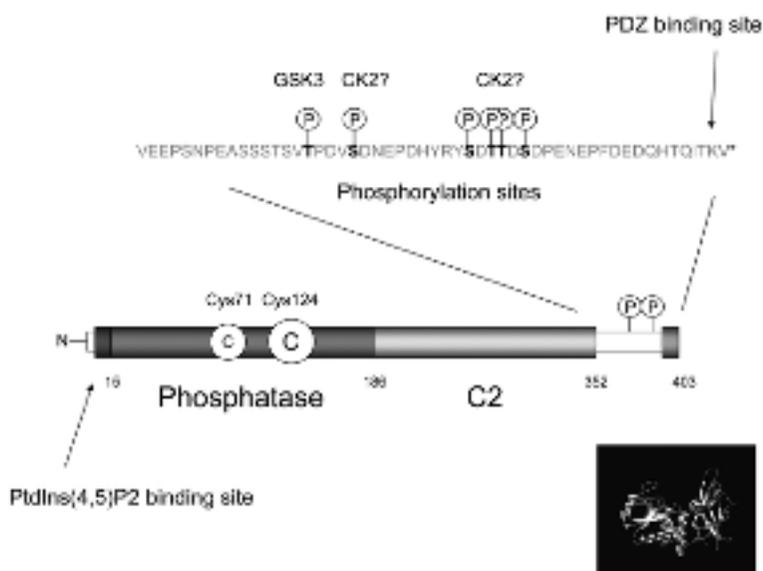


Figure 1 Domain structure of the PTEN protein. PTEN comprises a 403 amino acid protein and is a member of the large protein tyrosine phosphatase family. The roles of the catalytic cysteine (Cys¹²⁴) and Cys⁷¹ in reversible oxidation of PTEN are discussed in the text. The catalytic domain contains the classical CX₅R signature motif of this family. The structure mainly consists of two domains, the catalytic domain itself and a Ca²⁺-independent C2 domain, both of which are required for enzymatic activity. The short N-terminus contains a basic patch which can bind to PtdIns(4,5)P₂ in membranes while the C-terminal tail contains multiple serine and threonine residues that can be phosphorylated by CK2. Thr³⁶⁶ is a newly identified target for phosphorylation by glycogen synthase kinase 3.

It also may explain why control of PTEN often involves its inhibition, providing a means to stimulate signalling without direct activation of PI3Ks.

Inositol lipids and phosphates as PTEN substrates

PTEN can utilize a variety of substrates *in vitro*. These include acidic phospho-tyrosyl peptides, inositol phosphates, such as $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,3,4,5,6)P_5$, and all naturally occurring inositol phospholipids that possess a phosphate in the 3-position of the inositol ring [2,6]. The substrate specificity of PTEN *in vivo* is thus a key to understanding the biological significance and tumour suppressor function of this enzyme. The crystal structure of PTEN [7] reveals a broad and deep active-site pocket with positively charged residues that bind and accommodate the bulky, negatively charged headgroup of $\text{PtdIns}(3,4,5)P_3$. Moreover, there is no indication from the structure of any likely interactions with either the glycerol or fatty acid moieties of its lipid substrate. Hence it is not surprising that, under some assay conditions, $\text{Ins}(1,3,4,5)P_4$ and $\text{Ins}(1,3,4,5,6)P_5$ are efficient PTEN substrates [6,8]. Since both of these compounds occur naturally in cells they could be important physiological substrates and/or could compete with lipid substrates for PTEN's attention. It appears, however, that PTEN's catalytic mechanism, in concert with allosteric regulation by the non-substrate lipid, $\text{PtdIns}(4,5)P_2$, prevent access of soluble substrates under physiological conditions.

A model for interfacial catalysis, allosteric regulation and substrate specificity of PTEN

PTEN interacts with acidic membranes or phospholipid vesicles via its Ca^{2+} -independent C2 domain, which, together with the phosphatase domain, comprises the minimal catalytic unit [7]. These interactions are enhanced by $\text{PtdIns}(4,5)P_2$ which interacts with a basic patch at the N-terminus of PTEN [4,9,10]. Indeed, $\text{PtdIns}(4,5)P_2$ was reported to stimulate PTEN's lipid phosphatase activity by an allosteric mechanism [11]. We disputed this claim because $\text{PtdIns}(4,5)P_2$ simultaneously augments lipid phosphatase while profoundly inhibiting activity against $\text{Ins}(1,3,4,5)P_4$, and argued instead that the active site of PTEN is not available to soluble substrates when the enzyme is adsorbed to lipid surfaces [2,9]. This argument also proves to be too simplistic because Campbell et al. [11] used water soluble, short-chain lipids in their assays that do not form micelles or vesicular aggregates at the concentrations used in the assays. Hence it seems that $\text{PtdIns}(4,5)P_2$ may indeed be an allosteric regulator of PTEN that functions to enhance its association with substrate-containing membranes and induces a conformational state that somehow prevents access to soluble substrates. This effect is further enhanced by physiological levels of monovalent salts (KCl or NaCl) which also promote hydrolysis of lipid substrates, but reduce activity against $\text{Ins}(1,3,4,5)P_4$ [6].

It is particularly interesting that mutation of several residues in the C2 domain of PTEN (M-CBR3 mutant) reduce its ability to associate with membranes, greatly reducing biological activity when expressed in PTEN null

cells, but also enhancing activity against soluble substrates [12]. Expression of PTEN(M-CBR3) greatly reduced cellular levels of Ins(1,3,4,5,6) P_5 , confirming that this mutant has improved access to soluble substrates *in vivo*. More recently we have also shown that phosphorylation of the C-terminal serine/threonine cluster (which appears to involve CK2, formerly known as casein kinase 2, in cells) induces a PTEN conformation that inhibits dephosphorylation of lipid substrates, but enhances metabolism of Ins(1,3,4,5) P_4 (C.P. Downes, N.R. Leslie and N. Perera, unpublished work). It therefore appears that in wild-type PTEN under physiological conditions, the enzyme adopts a conformation that restricts access to potentially competing soluble substrates. This effect is further enhanced by the allosteric actions of PtdIns(4,5) P_2 and by the C2 domain binding to membrane surfaces and reversed by phosphorylation of the C-terminus. These data are compatible with a two-state model in which PTEN exists in two conformations, one favouring lipid substrates over inositol phosphates and the other having a relatively greater activity against soluble substrates. This is illustrated in Figure 2 which also emphasizes the fact that physiological intracellular conditions favour the stabilization of PTEN in a form which greatly prefers lipid substrates.

As noted above, PTEN is capable of metabolizing several lipid substrates which possess a phosphate group at the 3-position of the inositol ring, but our evidence suggests that of these PtdIns(3,4,5) P_3 is the only physiologically relevant substrate *in vivo*. PTEN is an interfacial enzyme that binds relatively weakly to membrane surfaces by virtue of its C2 domain and N-terminal PtdIns(4,5) P_2 binding motif [7,9,13]. A detailed interfacial kinetic analysis confirmed that recombinant PTEN binds only weakly to the surface of lipid vesicles and enabled us to determine the values of K_{cat} and interfacial K_m (the mole fraction of substrate lipid or interfacial concentration required for half maximal activity) for several lipid substrates. Importantly, PTEN has a much lower interfacial K_m for PtdIns(3,4,5) P_3 than for other 3-phosphoinositides, indicating that it efficiently metabolizes only this lipid at the very low mole fractions that occur naturally in membranes [6].



Figure 2 A two-state model of PTEN-dependent catalytic function.

The model envisages two conformational states of PTEN that can be distinguished experimentally by the relative activities towards phospholipid vesicular substrates compared with soluble inositol phosphates. Factors that influence the stability of these putative states are illustrated and are discussed in detail in the text.

There is much debate in the literature on lipid metabolizing enzymes about whether these enzymes derive kinetic advantage by being targeted to substrate-bearing membranes and/or through interfacial activation [14]. The latter case implies that interfacial binding induces a high activity conformational state of the enzyme. As described above there is increasing evidence that PTEN undergoes interfacial activation that is characterized by a state that greatly prefers lipid substrates. This conclusion is further supported by the kinetic data which suggest, from the combination of K_{cat} and the interfacial binding constant, that PTEN can perform one or at best a handful of catalytic cycles each time it binds to the membrane. These 'brief encounters' with the membrane have recently been elegantly demonstrated in *Dictyostelium* and mammalian cells by total internal reflection fluorescence microscopy of GFP (green fluorescent protein)-tagged PTEN constructs [15].

Combining the above information a picture emerges of an enzyme which undergoes dynamic, transient interactions with membranes via its C2 domain and N-terminal PtdIns(4,5) P_2 binding site. These interactions induce a conformational state of the enzyme which is characterized by very high activity towards lipid substrates and correspondingly weak activity towards soluble inositol phosphates. In this state, differences in the values of interfacial K_m account for the very high degree of selectivity for PtdIns(3,4,5) P_3 over other 3-phosphorylated lipids. Current evidence suggests that PtdIns3P and probably PtdIns(3,5) P_2 are instead metabolized *in vivo* by members of the myotubularin family of lipid 3-phosphatases [16,17], while the major route of PtdIns(3,4) P_2 metabolism is its dephosphorylation to PtdIns3P by the inositol polyphosphate 4-phosphatases [18].

Inhibitory regulation of PTEN

PtdIns(3,4,5) P_3 , the product of type I PI3K is metabolized by two distinct routes. In addition to removal of the 3-phosphate by PTEN, several members of the inositide 5-phosphatase family have the capacity to degrade PtdIns(3,4,5) P_3 to PtdIns(3,4) P_2 . SHIP (SH2 domain containing inositol polyphosphate 5-phosphatases) 1 and 2 are the best characterized of these activities [19]. As both PTEN and SHIPs function to remove PtdIns(3,4,5) P_3 and SHIP2 has a similarly broad tissue distribution, it is perhaps surprising that PTEN and SHIP2 appear to have very different biological roles. Whereas PTEN homozygous knockout is embryonic lethal in mice [20], SHIP2^{-/-} mice are viable and either die from extreme hypoglycaemia or are resistant to the effects of a high fat diet, making SHIP2 an attractive target for Type II diabetes or treatment of problems resulting from the so-called metabolic syndrome [21,22]. One difference is that PTEN directly reverses the PI3K reaction whereas SHIPs generate another potential lipid second messenger, PtdIns(3,4) P_2 . The latter lipid binds to several proteins that are also targets of PtdIns(3,4,5) P_3 , such as PKB (protein kinase B) and DAPP (Dual Adaptor for Phosphoinositides and Phosphotyrosine)1, but also binds highly selectively to the PH (pleckstrin homology) domain of TAPP (Tandem PH domain protein)1 [23]. TAPP1 in turn binds to the PTP (protein tyrosine phosphatase) L1, and this complex translocates to the plasma membrane

when PtdIns(3,4) P_2 levels are elevated. It is possible that this provides a feedback loop whereby growth factor receptor-stimulated tyrosine kinase activity can be antagonized [24]. The actin regulatory protein, MIG-10/lamellipodin, also possesses a PH domain with reported specificity for PtdIns(3,4) P_2 , implicating this lipid in axon guidance during neuronal development [25,26].

While the signalling function of PtdIns(3,4) P_2 may explain in part the differences between PTEN and SHIP2-mediated metabolism of PtdIns(3,4,5) P_3 , there is another important distinction between these pathways. SHIPs contain an SH2 domain, a poly-proline region and phosphotyrosine motifs, and appear to be tightly regulated, with low basal cellular activity. By contrast, PTEN lacks obvious regulatory domains and appears to have relatively high constitutive activity *in vitro* and in cells. This would explain why PTEN null cells have greatly elevated PtdIns(3,4,5) P_3 levels and thus constitutive activation of PI3K signalling pathways and also prompts the question as to whether PTEN may normally be subject to negative regulation.

In our laboratory we have been exploring this idea by studying regulatory mechanisms that inhibit PTEN activity. The first such mechanism to be characterized in detail involves the reversible oxidation of PTEN's active-site cysteine residue (see Figure 1). PTEN is a member of the protein tyrosine phosphatase superfamily which relies on a highly reactive active site cysteine residue in the characteristic CX₅R motif. PTEN has been shown to be highly sensitive to oxidation, and in response to treatment with hydrogen peroxide forms an inactive disulfide involving the active-site residue (Cys¹²⁴) and Cys⁷¹ which lie close together in the crystal structure [27]. This process can be reversed in the presence of reducing agents which restore the enzyme to full activity. We were able to show that cellular PTEN can be oxidized both by exposure of cells to exogenous hydrogen peroxide or by production of endogenous oxidants in response to receptor stimulation. Moreover, oxidants could activate PI3K signalling, as indicated by the phosphorylation and activity state of PKB, in a PTEN-dependent manner [28]. More recently, PDGF (platelet-derived growth factor) and insulin were shown to stimulate oxidation of a fraction of the cellular PTEN with consequent effects on the PI3K pathway [29,30]. These results provide a potential mechanism for the well-known insulin-mimetic effects that occur on exposure of many cells to hydrogen peroxide. It is possible that reversible oxidation of PTEN, and perhaps several other members of the protein tyrosine phosphatase family, will prove to be a control mechanism which bears comparison with other post-translational modifications such as phosphorylation of serine, threonine or tyrosine residues. If so then it will be necessary in each case to define the relevant oxidizing and reducing species in the cellular environment which are responsible for the forward and reverse reactions in such a process.

The above results suggest that inhibition of PTEN provides a means to stimulate downstream signalling through the basal, unstimulated activity of PI3K. More significantly it may provide a feed forward mechanism that would amplify PI3K signalling in a spatially coordinated manner. This is because PtdIns(3,4,5) P_3 can activate guanine nucleotide exchange factors that convert the small GTPase, Rac, into its active, GTP-bound form and this in turn can participate in the assembly of an active NADPH oxidase which synthesizes

superoxide anion at the plasma membrane [31]. The generation of short-lived reactive oxygen species at the site of active PtdIns(3,4,5) P_3 production should locally oxidize and inhibit PTEN thus amplifying the signal. It seems likely that reversible oxidation of PTEN could provide a means to generate the steep gradients of PtdIns(3,4,5) P_3 that are required for polarized cell responses such as cell movement in a chemo-attractant gradient [32].

Is PTEN a dual specificity enzyme with biologically significant protein tyrosine phosphatase activity?

This is a question that has intrigued the field since the discovery of the PTEN gene as a major tumour suppressor [33,34]. It was immediately recognized that the gene encodes a protein that is a member of the protein tyrosine phosphatase family and many attempts were made to define its substrate preferences and to identify cellular protein substrates. It was discovered that PTEN prefers highly acidic phosphotyrosyl-peptide substates such as poly(Glu-Tyr-P), but at this stage its physiological substrates remained obscure. The discovery that PTEN specifically removes the 3-phosphate from the inositide substrates discussed above was an important breakthrough [35] and was quickly followed by the observation that a mutation found in a Cowden's disease patient (G129E), which retained wild-type protein tyrosine phosphatase activity, was devoid of lipid phosphatase activity. This made it clear that the lipid phosphatase activity of PTEN is required for many of PTEN's biological functions and at least some if not all of its tumour suppressor activity [36].

With a clear picture emerging of the roles of PTEN as an antagonist of PI3K signalling, attention has turned once more to the biological significance of its protein tyrosine phosphatase activity. Most of the work in this area makes use of PTEN(G129E), comparing its biological effects either with C124S or G129R mutations, both of which lack all enzyme activity. One of the first indications of a biological function for PTEN's protein tyrosine phosphatase activity came from studies of tumour cell migration which could be efficiently blocked using PTEN(G129E), but not phosphatase-inactive mutants [37]. FAK (focal adhesion kinase) and the adaptor protein, Shc were proposed as tyrosine phosphorylated PTEN substrates, but subsequent work failed to confirm this [38–40]. More recently several important biological responses have been attributed to PTEN's protein tyrosine phosphatase activity. These include inhibition of ERK (extracellular-signal-regulated kinase) phosphorylation and activation, effects of PTEN on up or downregulation of expression of several genes and/or proteins [e.g. downregulation of expression of A kinase adaptor protein 121, downregulation of IGF (insulin growth factor)-II expression in hepatoma cells and inhibition of hyaluronic acid induced matrix metalloproteinase-9 expression] and inhibition of orthotopic invasion of human bladder cancer cells. The putative PTEN protein substrates in each of these cases were not identified [41–45].

As discussed in the preceding sections, the efficiency of PTEN as a lipid phosphatase depends upon interfacial localization and activation mechanisms.

It might be expected therefore, that physiologically relevant protein substrates of PTEN might physically interact either with PTEN itself or through common scaffold proteins. PTEN is known to interact with multi PDZ-domain proteins of the MAGI (membrane-associated guanylate kinase with inverted domain structure) family via its C-terminus (see Figure 1). Whether PTEN targets any direct protein substrates in this way is not clear at present although β -catenin and the PDGF (platelet-derived growth factor) receptor are two possibilities [46,47]. In the latter case, PTEN binds to one or both of the PDZ domains of the Na⁺/H⁺ exchange regulatory factor (NHERF) proteins which in turn can form a ternary complex with the PDGF receptor [48].

PTEN may also interact directly with the 5-HT_{2C} receptor (5-HT is 5-hydroxy-tryptamine) via the latter's third intracellular loop and thereby antagonize agonist-induced phosphorylation. Disruption of this interaction mimicked the action of a 5-HT_{2C} receptor agonist by suppressing the enhanced firing rate of dopaminergic neurons induced by cannabinoids [49]. Another receptor that appears to bind PTEN directly is the NMDA (*N*-methyl-D-aspartate) receptor, the function of which depends upon PTEN's protein phosphatase activity, although the precise mechanism is not yet understood [50].

Despite the recent progress in this area, much more work is needed to confirm the above results and to establish firmly the simple enzyme/substrate relationships that are implied by mostly indirect studies.

A phosphatase-independent function of PTEN?

Significant progress has been made recently in understanding the molecular basis by which PTEN inhibits cell migration, one of the earliest observed effects attributable to PTEN's protein tyrosine phosphatase activity (see above), and these studies also identify a phosphatase-independent function of the C2 domain [51]. PTEN is known to play an important role in the regulation of cell migration in the slime mould, *Dictyostelium discoideum* [14], and in mammalian cells. Despite the fact that PI3K signalling contributes to cell motility and chemotaxis, the ability of PTEN to prevent tumour cell migration can be attributed to its protein tyrosine phosphatase activity since PTEN (G129E) is fully active in such models. Hall and colleagues [51] recently made use of an *in vitro* 'wound healing' assay to study cell migration in human glioma cell lines. These cells were PTEN null and, not surprisingly, re-expression of PTEN efficiently blocked migration of cells into the 'wound'. The effects of wild-type PTEN were fully reproduced by PTEN(G129E), but not by a phosphatase-dead mutant, implying that the protein tyrosine phosphatase activity of the enzyme was sufficient to prevent cell migration in this model. However, it was found that constructs lacking all phosphatase activity, but containing only the C2 domain also effectively prevented cell migration. This surprising result implied that the potential effect of the C2 domain must somehow be suppressed in the full-length protein and that the protein phosphatase activity of PTEN may be required to expose this function. Further, indirect experiments suggested that the phosphorylated C-terminus of PTEN could suppress the function of the C2 domain and that

autodephosphorylation, specifically of Thr³⁸³, was sufficient to relieve this inhibitory effect. This ability of the C2 domain to block cell migration was specific to PTEN because C2 domains from two other proteins were ineffective. Nevertheless these experiments do rely on overexpression of tagged proteins, and the molecular basis of the effect of the C2 domain remains unclear.

Further evidence for a phosphatase-independent function of PTEN has come from studying the PTEN interacting protein MSP58 [52]. When identified in a yeast 2-hybrid screen, this protein appeared interesting, as it had been previously shown to transform chicken embryo fibroblasts. The authors went on to show that MSP58 bound to PTEN through what appears to be a phospho-specific interaction with phospho-Thr³⁶⁶ of PTEN. The functional significance of this interaction was implied by transformation assays in which anchorage-independent growth of mouse embryo fibroblasts was induced by MSP58 over-expression, but could be inhibited by either a wild-type or phosphatase-dead form of PTEN.

We decided to study the effects of PTEN on cell migration in a completely different and arguably more physiological context in the chicken embryo during gastrulation (C.P. Downes, N.R. Leslie, C. J. Weijer and X. Yang, unpublished work). In these experiments we are examining the migration of cells from the anterior primitive streak out into the mesoderm and back to line the length of the developing primitive streak. We also found that PTEN(G129E) was fully effective in blocking cell migration in this model. This effect, however, was abolished by removing the extreme C-terminus containing the PDZ domain binding motif implying that the targeting of PTEN through interaction with scaffold proteins is required. A current understanding of the mechanism underlying PTEN's ability to prevent cell migration is illustrated in Figure 3.

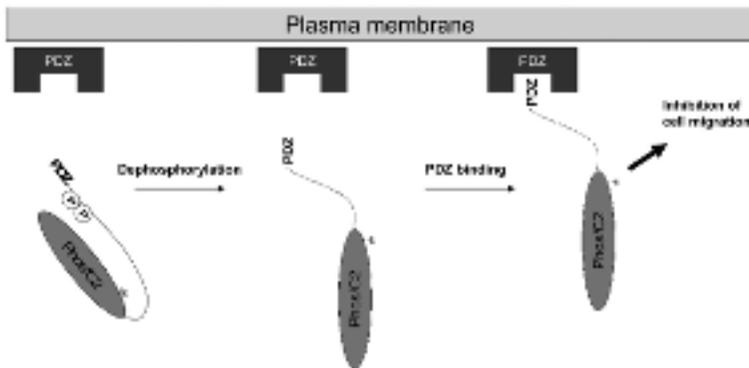


Figure 3 Mechanisms involved in PTEN-dependent inhibition of cell migration. The model envisages that PTEN exists in a basal, closed state in which the C-terminal cluster of phosphorylated residues prevents interactions of both the C2 domain and PDZ domain binding motif with their targets. Autodephosphorylation of Thr³⁸³ either alone or accompanied by further dephosphorylation of other sites in the cluster opens up the structure allowing PTEN to bind to PDZ domain-containing scaffold proteins and the C2 domain to participate in the observed inhibition of cell migration.

Conclusions

PTEN is a dual specificity phosphatase that exerts much of its biological influence through its ability to antagonize PI3K signalling by selectively hydrolysing the 3-phosphate of the lipid second messenger, PtdIns(3,4,5) P_3 . The evidence suggests that PTEN is constitutively active in cells and has a key role in maintaining basal PtdIns(3,4,5) P_3 levels below the signalling threshold. Hence negative regulation of PTEN, inactivating mutations, or reduced expression of the enzyme can all activate signalling pathways either independently of or in concert with stimulation of PI3K activity. The high degree of selectivity of PTEN *in vivo* is remarkable given its active site architecture and appears to be explained by the interfacial activation of the enzyme and more subtle discrimination between the different 3-phosphoinositide substrates that is reflected in the values for interfacial K_m . The current interest in PTEN's protein phosphatase activity needs to bear these points in mind. For example, it seems likely that heterologous protein substrates would need to be specifically co-localized and/or orientated to the active site in order for PTEN to function as an efficient phosphatase. Although much progress has been made using PTEN(G129E) that lacks lipid phosphatase activity, but retains activity against model peptide substrates, the identification of mutations with the opposite selectivity (i.e. retaining lipid phosphatase, but not protein phosphatase activity) would provide valuable tools to determine the biological significance of PTEN's apparent dual specificity.

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References

1. Waite, K.A. and Eng, C. (2002) *Am. J. Hum. Genet.* **70**, 829–844
2. Lelsie, N.R. and Downes, C.P. (2002) *Cell. Signalling* **14**, 285–295
3. Simpson, L. and Parsons, R. (2001) *Exp. Cell Res.* **264**, 29–41
4. Maehama, T., Taylor, G.S. and Dixon, J.E. (2001) *Annu. Rev. Biochem.* **70**, 247–279
5. Seminario, M.C. and Wange, R.L. (2003) *Immunol. Rev.* **192**, 80–97
6. McConnachie, G., Pass, I., Walker, S.M. and Downes, C.P. (2003) *Biochem. J.* **371**, 947–955
7. Lee, J.O., Yanf, H., Georgescu, M.M., Di cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P. and Pavletich, M.P. (1999) *Cell* **99**, 323–334
8. Caffrey, J.J., Darden, T., Wenk, M.R. and Shears S.B. (2001) *FEBS Lett.* **499**, 6–10
9. Walker, S.M., Leslie, N.R., Perera, N.M., Batty, I.H. and Downes, C.P. (2004) *Biochem. J.* **379**, 301–307
10. Lemmon, M.A. and Ferguson, K.M. (2000) *Biochem. J.* **350**, 1–18
11. Campbell, R.B., Liu, F. and Ross, A.H. (2003) *J. Biol. Chem.* **278**, 33617–33620
12. Orchiston, E.A., Bennett, D., Leslie, N.R., Clarke, R.G., Winward, L., Downes, C.P. and Safrany, S.T. (2004) *J. Biol. Chem.* **279**, 1116–1122
13. Das, S., Dixon, J. E. and Cho, W. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 7491–7496
14. Carmen, G.M., Deems, R.A. and Dennis, E.A. (1995) *J. Biol. Chem.* **270**, 18711–18714
15. Vazquez, F., Matsuoka, S., Sellers, W.R., Yanagida, T., Ueda, M. and Devreotes, P.N. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103**, 3633–3638
16. Taylor, G.S. and Dixon, J.E. (2003) *Methods Enzymol.* **366**, 43–56
17. Tronchere, H., Laporte, J., Pendaries, C., Chaussade, C., Liaubet, L., Pirola, L., Mandel, J.L. and Payrastre, B. (2004) *J. Biol. Chem.* **279**, 7304–7312

18. Ivetac, I., Munday, A.D., Kisseleva, M.V., Zhang, X.M., Luff, S., Tiganis, T., Whisstock, J.C., Rowe, T., Majerus, P.W. and Mitchell, C.A. (2005) *Mol. Biol. Cell* **16**, 2218–2233
19. Backers, K., Blero, D., Paternotte, N., Zhang, J. and Erneux, C. (2003) *Adv. Enzyme Regul.* **43**, 15–28
20. Kishimoto, H., Hamada, K., Saunders, M., Backman, S., Sasaki, T., Nakano, T., Mak, T.W. and Suzuki, A. (2003) *Cell Struct. Funct.* **28**, 11–21
21. Clement, S., Krause, U., Desmedt, F., Tanti, J.F., Behrends, J., Pesesse, X., Sasaki, T., Penninger, J., Doherty, M., Malaisse, W. et al. (2001) *Nature* **409**, 92–97
22. Sleeman, M.W., Wortley, K.E., Lai, K.M., Gowen, L.C., Kintner, J., Kline, W.O., Garcia, K., Stitt, T.N., Yancopoulos, G.D., Wiegand, S.J. and Glass, D.J. (2005) *Nat. Med.* **11**, 199–205
23. Dowler, S., Currie, R.A., Downes, C.P. and Alessi, D.R. (1999) *Biochem. J.* **342**, 7–12
24. Kimber, W.A., Deak, M., Prescott, A.R. and Alessi, D.R. (2003) *Biochem. J.* **376**, 525–535
25. Krause, M., Leslie, J.P., Stewart, M., Lafuente, E.M., Valderrama, F., Jagannathan, R., Strasser, G.A., Robinson, D.A., Liu, H., Way, M. et al. (2004) *Dev. Cell* **7**, 571–583
26. Adler, C.E., Fetter, R.D. and Bargmann, C.I. (2006) *Nat. Neurosci.* **9**, 511–518
27. Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W. and Rhee, S.G. (2002) *J. Biol. Chem.* **273**, 15366–15372
28. Leslie, N.R., Bennett, D., Lindsay, Y.E., Stewart, H., Gray, A. and Downes, C.P. (2003) *EMBO J.* **22**, 5501–5510
29. Kwon, J., Lee, S.R., Yang, K. S., Ahn, Y., Kim, Y.J., Stadtman, E.R. and Rhee, S.G. (2004) *Proc. Natl. Acad. Sci. U.S.A.* **101**, 16419–16424
30. Seo, J.H., Ahn, Y., Lee, S.R., Yeol Yeo, C. and Chung Hur, K. (2005) *Mol. Biol. Cell* **16**, 348–357
31. Welch, H.C., Condliffe, A.M., Milne, L.J., Ferguson, G.J., Hill, K., Webb, M.L., Okkenhaug, K., Coadwell, W.J., Andrews, S.R., Thelen, M. et al. (2005) *Curr. Biol.* **15**, 1867–1873
32. Weiner, O.D., Neilsen, P.O., Prestwich, G.D., Kirschner, M.W., Cantley, L.C. and Bourne, H.R. (2002) *Nat. Cell Biol.* **4**, 509–513
33. Li, J., Liaw, D., Podyspyanina, K., Bose, S., Wang, S.I., Puc, J., Miliareisis, C., Rodgers, L., McCombie, R., Bigner, S.H. et al. (1997) *Science* **275**, 1943–1947
34. Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T. et al. (1997) *Nat. Genet.* **15**, 356–362
35. Maehama, T. and Dixon, J.E. (1998) *J. Biol. Chem.* **273**, 13375–13378
36. Myers, M.P., Pass, I., Batty, I.H., van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P. and Tonks, N.K. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13513–13518
37. Tamura, M., Gu, J. and Yamada, K.M. (1998) *Science* **280**, 1614–1617
38. Gu, J., Tamura, M., Pankov, R., Danen, E.H., Takino, T., Matsumoto, K. and Yamada, K.M. (1999) *J. Cell Biol.* **146**, 389–404
39. Weng, L.P., Brown, J.L., Baker, K.M., Ostrowski, M.C. and Eng, C. (2002) *Hum. Mol. Genet.* **11**, 1687–1696
40. Liliental, J., Moon, S.Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H. and Wu, H. (2000) *Curr. Biol.* **6**, 401–404.
41. Abounader, R., Reznik, T., Colantuoni, C., Martinez-Murillo, F., Rosen, E.M. and Laterra, J. (2004) *Oncogene* **23**, 9173–9182
42. Huang, Y., Wernyj, R.P., Norton, D.D., Precht, P., Seminario, M.C. and Wange, R.L. (2005) *Oncogene* **24**, 3819–3829
43. Kang-Park, S., Lee, Y.I. and Lee, Y.I. (2003) *FEBS Lett.* **545**, 203–208
44. Park, M.J., Kim, M.S., Park, I.C., Kang, H.S., Yoo, H., Park, S.H., Rhee, C.H., Hong, S.I. and Lee, S.H. (2002) *Cancer Res.* **62**, 6318–6322
45. Gildea, J.J., Herlevsen, M., Harding, M.A., Gulding, K.M., Moskaluk, C.A., Frierson, H.F. and Theodorescu, D. (2004) *Oncogene* **23**, 6788–6797
46. Vogelmann, R., Nguyen-tat, M.D., Giehl, K., Wedlich, D. and Menke, A. (2005) *J. Cell Sci.* **118**, 4901–4912
47. Mahimainathan, L. and Choudhury, G.G. (2004) *J. Biol. Chem.* **279**, 15258–15268
48. Takahashi, Y., Morales, F.C., Kreimann, E.L. and Georgescu, M.M. (2006) *EMBO J.* **25**, 910–920
49. Ji, S.P., Zhang, Y., Cleemput, J.V., Jiang, W., Liao, M., Li, L., Wan, Q., Backstrom, J.R. and Zhang, X. (2006) *Nat. Med.* **12**, 324–329

50. Ning, K., Pei, L., Liao, M., Liu, B., Zhang, Y., Jiang, W., Mielke, J.G., Li, L., Chen, Y., El-Hayek, Y.H. et al. (2004) *J. Neurosci.* **24**, 4052–4060
51. Raftopoulou, M., Etienne-Manneville, S., Self, A., Nicholls, S. and Hall, A. (2004) *Science* **303**, 1179–1181
52. Okumura, K., Zhao, M., DePinho, R.M., Furnari, F.B. and Cavenee, W.K. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 2703–2706