Antagonism of PI 3-kinase-dependent signalling pathways by the tumour suppressor protein, PTEN
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Abstract
The tumour suppressor protein, PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a member of the mixed function, serine/threonine/tyrosine phosphatase subfamily of protein phosphatases. Its physiological substrates, however, are primarily 3-phosphorylated inositol phospholipids, which are products of phosphoinositide 3-kinases. PTEN thus antagonizes PI 3-kinase-dependent signalling pathways, which explains to a large extent its tumour suppressor status. We have examined the kinetic behaviour, substrate specificity and regulation of PTEN both in vitro and in a variety of cellular models. Although PTEN can utilize both phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3] and its water-soluble headgroup, inositol 1,3,4,5-tetraakisphosphate, as substrates, it displays classical features of interfacial catalysis, which greatly favour the lipid substrate (by as much as 1000-fold as judged by Km/K values). Expression of PTEN in U87 cells (which lack endogenous PTEN) and measuring the levels of all known 3-phosphorylated lipids suggests that phosphatidylinositol 3,4-bisphosphate and PtdIns(3,4,5)P3 are both substrates, but that phosphatidylinositol 3-phosphate and phosphatidylinositol 3,5-bisphosphate are not. PTEN binds to several PDZ-domain-containing proteins via a consensus sequence at its extreme C-terminus. Disruption of targeting to PDZ-domain proteins selectively blocks some PTEN functions, but not others, suggesting the existence of spatially localized, functionally dedicated pools of signalling lipids. We have also shown recently that PTEN expression is controlled at the transcriptional level and is profoundly upregulated by peroxisome proliferator-activated receptor γ agonists, thereby providing possible implications for these drugs in diabetes, inflammation and cancer.

Introduction
The are several compelling lines of evidence for the claim that PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a human tumour suppressor gene. It was first identified in 1997 as a locus that was mutated frequently in sporadic glioblastomas, prostate and breast cancers and that is located at chromosome 10q23 in a region that is deleted at very high frequency in...
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many tumour types [1,2]. It is now known that PTEN mutations are associated with a wide range of tumours, most frequently endometrial tumours and glioblastomas. Germline mutations in PTEN result in the uncommon autosomal dominant hamartomatous syndromes, Cowden disease and Bannayan–Ruvalcaba–Rily syndrome. Cowden disease sufferers experience a greatly increased risk of breast and thyroid tumours. The critical importance of PTEN during development is indicated by the fact that PTEN knockout mice die during embryogenesis between days 6.5 and 9.5. Mice that are heterozygous at the PTEN locus, however, develop normally, but are susceptible to a wide range of tumours later in life, similar to human Cowden disease patients. This evidence is summarized in several recent reviews [3–5].

The PTEN gene encodes a 403 amino acid protein (PTEN) that is a member of the protein tyrosine phosphatase (PTP) subfamily of protein phosphatases. Its primary physiological substrates, however, are 3-phosphorylated inositol phospholipids, which are products of phosphoinositide 3-kinases (PI 3-kinases). PTEN thus antagonizes PI 3-kinase-dependent signalling pathways, in large part explaining its tumour-suppressor status. The discovery that PTEN metabolizes 3-phosphoinositide lipid signals arose from the failure to identify convincing protein substrates, which culminated in the observation that the most promising peptide-based substrates contained multiple acidic residues. This led Tonks and co-workers to propose acidic phospholipids as possible substrates [6], an idea that was first addressed by Maehama and Dixon [7]. They established that PTEN metabolized phosphoinositide substrates in vitro and in cell lines, while Myers et al. [8] subsequently linked the ability to metabolize lipids with tumour suppressor activity using a mutant (G129E) that lacked lipid phosphatase activity, but retained phosphotyrosine phosphatase activity, and which occurs in Cowden disease patients.

The connections between PTEN, its lipid substrates, the signalling pathways that they control and tumour suppression are now well established. Much less is known about PTEN as a suppressor of insulin action or in inflammatory responses involving neutrophils and macrophages, two areas of biology in which PI 3-kinase-dependent signalling pathways have decisive roles [9–12]. Moreover, there is very little information on the factors and mechanisms that control the level of PTEN activity in cells. In this paper, we explore the kinetic behaviour of PTEN that contributes to its unexpected substrate specificity and identify two mechanisms of regulation involving spatial distribution of the enzyme [13,14] and enhanced transcription of the PTEN gene stimulated by peroxisome proliferator activated receptor (PPAR)γ agonists [15]. The latter results suggest a role for PTEN in inflammation and diabetes as well as cancer.

PTEN is a member of the protein tyrosine phosphatase family that is well adapted to utilizing phosphoinositide substrates

The crystal structure of PTEN was solved recently by Lee et al. [16]. As predicted from the primary sequence it contains an N-terminal phosphatase domain, but it also possesses several characteristics that are not shared by other PTP enzymes and that direct the phosphatase towards lipid substrates. For example, PTEN has a Ca²⁺-independent C2 domain that can bind phospholipid vesicles in vitro and appears not only to allow transient association with cellular membranes, but also to play a role in ensuring the productive orientation of the active site. This idea is supported by the extensive interface between the phosphatase domain and the catalytic domain that is highly conserved in PTEN orthologues from different species, and is also a frequent target of mutations.

Ribbon diagram showing the domain structure and architecture of PTEN

The N-terminal phosphatase domain is shown in blue, the C2 domain in red and both the N and C terminal positions are labelled. Tartrate, a weak competitive inhibitor, is shown as van der Waal’s spheres occupying the active site (carbon atoms are black, oxygens are red). The figure was produced by Dr C. Bond (Wellcome Trust Biocentre, Dundee) using MOLSCRIPT [27] and Protein Data Bank entry 1DSI [16].
in tumours. A further structural adaptation of PTEN involves the active site itself. The substrate-binding pocket in PTEN is both deeper and broader than in other PTPs for which structures have been determined, allowing access to the bulky polyphosphorylated inositol phospholipid headgroup. The cleft is also highly basic, consistent with the preference for acidic substrates. The domain structure of PTEN is illustrated in Figure 1.

Early studies established that PTEN selectively removes the 3-phosphate from the water-soluble inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P₄). Figure 2 illustrates the range of inositol phospholipids currently known to be present in eukaryotic cells and indicates 3-phosphorylated species that are potential substrates of PTEN. Our initial studies focused on phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃), the principal product of type I PI 3-kinases and an established mitogenic second messenger and insulin signalling molecule [17]. For the kinetic analyses, recombinant human PTEN was prepared as a glutathione-S-transferase fusion protein, that is cleaved from this tag by thrombin, and purified to homogeneity. Synthetic phosphoinositides were mixed with PtdIns(3,4,5)P₃ that had been labelled with ³²P at the 3-position (by the action of recombinant PI 3-

Figure 2

**Eukaryotic cells synthesize at least eight distinct phosphoinositide species – potential substrates of PTEN**

Phosphatidylinositol is synthesized by the combination of CMP-phosphatidate (CMP-PA) and myo-inositol (Ins). Almost 50 years of work on these compounds has identified the seven polyphosphoinositide species found in eukaryotic cells, which are shown in this figure. These differ in terms of the number and distribution of monoester phosphate groups around the six-membered inositol ring. The arrows indicate known pathways of synthesis and degradation of these molecules. Question marks indicate putative dephosphorylation reactions catalysed by PTEN which are addressed in the text. PtdIns(3,4,5)P₃ is currently the only confirmed substrate of PTEN in living cells.
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Specific targeting of PTEN to PDZ-domain protein scaffolds is impaired in a subset of human tumours

Although PTEN appears to be located mainly in the cytosol of most cells, the extreme C-terminus contains a consensus sequence that mediates interactions with a number of PDZ domains in several proteins. Of the proteins that bind PTEN in vitro and in overexpression studies, MAGI-2 is so far the only one for which an interaction with the endogenous protein has been demonstrated [20]. In our experiments we deleted the PDZ domain binding sequence (generating PTENstop), a strategy that has no measurable effect on the catalytic activity of PTEN, and analysed the effects on a range of cellular responses to PTEN expression. This manipulation had no detectable effect on the ability of PTEN to inhibit protein kinase B (PKB) activity, signalling components downstream of PKB or the PKB-independent activation of p70S6 kinase in PTEN-null U87 tumour cells. By contrast, this mutation severely blunted the inhibitory effect of PTEN expression on cell spreading in U87 cells and platelet-derived growth factor-induced membrane ruffling in Swiss 3T3 fibroblasts [13,14], suggesting that PDZ domain interactions are required for efficient inhibition of lamellipodia formation. This observation is particularly intriguing because the effects on lamellipodia required the lipid phosphatase activity of PTEN, but the expression of the PTENstop mutation made no detectable difference to the decrease in PtdIns(3,4,5)P3 levels that occurred in U87 cells, compared with the effect of wild-type PTEN. It seems, therefore, that PDZ domain-mediated targeting of PTEN may play a role in the regulation of processes that are highly sensitive to small changes in the level of PtdIns(3,4,5)P3. This in turn suggests the possibility that a small localized pool of PtdIns(3,4,5)P3 is dedicated to regulating lamellipodia formation, but not the activation of PKB. We are currently seeking direct evidence for the involvement of dedicated pools of signalling lipids in the regulation of spatially localized cellular responses.

These data were also extended to include an analysis of tumours carrying mutations in the extreme C-terminus of PTEN, similar to the PTENstop mutation that we constructed. These experiments showed relatively low levels of PKB phosphorylation in a glioblastoma sample that carried such a C-terminal mutation compared with tumours carrying phosphatase-inactivating mutations of the enzyme [14]. This suggests that deregulation of PKB is not a universal feature of tumours that carry PTEN mutations. We postulate that the deficiency in these tumours reflects a failure to target PTEN to ensure efficient metabolism of a small PKB-independent pool of

interaction with lipid substrates owing to the completion of multiple catalytic cycles at each encounter with the substrate-bearing surface. A diagnostic feature of such enzymes acting on vesicular substrates is that initial velocity increases when the bulk concentration of substrate is held constant, but the mole fraction is increased. In such an experiment, as the mole fraction is increased, fewer vesicles are added to keep the bulk substrate concentration the same in each assay [19]. Such an effect is readily observed for PTEN acting on PtdIns(3,4,5)P3 in vesicles composed primarily of PtdCho. It is also possible to determine an apparent value of \( V_{\text{max}} \) for each of several mole fractions of substrate. A plot of \( V_{\text{max}} \) (apparent) versus mole fraction is then used to derive the interfacial \( K_m \), i.e. the mole fraction of substrate required to yield a value of \( V_{\text{max}} \) that is half of the value obtained at a high, 'saturating' mole fraction of substrate. This type of analysis suggests a remarkably low interfacial \( K_m \) value for PTEN of less than 0.1 \( \% \). Put simply, this means that PTEN is an incredibly efficient interfacial enzyme that is ideally suited to metabolizing lipids that comprise a very small proportion of the total membrane lipid content. Unstimulated levels of PtdIns(3,4,5)P3 are of the order of 0.001 \( \% \) of total phospholipid, but much higher concentrations are expected around signalling complexes containing activated PI 3-kinases at the plasma membrane. Mutational analyses are under way to identify the sequence motifs and structural features required for efficient interfacial catalysis of PtdIns(3,4,5)P3 hydrolysis.

Further experiments along similar lines to those described above suggest a substrate order of preference of PtdIns(3,4,5)P3 > phosphatidylinositol 3,4-bisphosphate > phosphatidylinositol 3-phosphate. Moreover, experiments in which PTEN was overexpressed in a PTEN-null tumour cell line suggest that neither phosphatidylinositol 3-phosphate nor phosphatidylinositol 3,5-bisphosphate are cellular substrates of the expressed enzyme. This might be a reflection of their relative weakness as substrates in vitro, lack of accessibility of some potential substrates in intact cells, or both.
PtdIns(3,4,5)P₃. Approx. 5% of PTEN mutations in glioblastomas are on the C-terminal side of the C2 domain, but no such mutations occur in endometrial tumours. This might reflect the finding that PTEN mutation is an early event in endometrial tumour progression, but is associated with advanced malignant brain tumours [21].

**Cellular PtdIns(3,4,5)P₃ levels are controlled via upregulation of PTEN gene transcription**

Although PTEN exists as a phosphoprotein in cells, there is no evidence for signalling pathways that regulate PTEN activity through acute changes in phosphorylation. Phosphorylation of a cluster of serine and threonine residues in the C-terminal tail of PTEN (possibly via casein kinase 2) appears to be important for stability of the enzyme presumably by inhibiting its degradation by a proteosome-mediated pathway [22,23]. Since these sites appear to be constitutively phosphorylated in cells it will be interesting to identify the protein phosphatase(s) that are responsible for dephosphorylation, since activation of such a pathway would be expected to dephosphorylate PTEN and perhaps target it for degradation. Such a mechanism could possibly account for the dramatic downregulation of PTEN expression that occurs in human keratinocytes treated with transforming growth factor β [24].

Recent work has started to highlight the importance of transcriptional regulation of PTEN gene expression. Analysis of the putative promoter region upstream of the PTEN transcriptional start-site revealed the presence of two possible response elements for PPARγ within 23.3 kb of sequence as illustrated in Figure 3. PPARγ is a member of the ligand-activated nuclear receptor superfamily. Although there is currently much debate about the identities of natural ligands for this receptor, synthetic ligands, such as rosiglitazone, are proving useful in the treatment of type II diabetes. Recently PPARγ has been shown to regulate differentiation and/or cell growth in many cell types leading to the suggestion that this receptor, like PTEN, may function as a tumour suppressor. Moreover, PPARγ is also implicated in anti-inflammatory responses, but mechanisms underlying these varied roles remain ill-defined. We have shown that activation of PPARγ by rosiglitazone dramatically upregulates PTEN expression in human macrophages, Caco2 colorectal cancer cells and MCF7 breast cancer cells [15].

Upregulation is observed at the mRNA, protein and phosphoinositide 3-phosphatase activity levels by a mechanism that most likely involves the direct interaction of activated PPARγ with one or more of the response elements noted above, although more work needs to be done to confirm this.

These results suggest that upregulation of PTEN, with the concomitant downregulation of PI 3-kinase-dependent signalling pathways, might be one of the mechanisms through which PPARγ agonists exert their anti-inflammatory and anti-cancer actions. There is an apparent paradox, however, in the efficacy of these compounds in the treatment of type II diabetes, in which further blunting of PI 3-kinase signalling would be expected to be detrimental. However, it seems that overexpression of PTEN elicits a feedback mechanism that results in the upregulation of insulin receptor substrate 2 (IRS-2), one of a family of at least four adapter proteins through which insulin and some other stimuli activate PI 3-kinase [25]. Since transgenic IRS-2-/- mice exhibit many features of type II diabetes [26], it is likely that the upregulation of IRS-2 seen with enhanced expression of PTEN will be beneficial in this disease.

**Conclusions**

We have established kinetic properties of PTEN that reveal its efficiency as an enzyme for metabolizing a lipid signal that is a very minor constituent of cell membranes. These studies also suggest, not only its remarkable adaptation as a lipid phosphatase, but also the very high degree of specificity towards PtdIns(3,4,5)P₃ as the physiologically relevant substrate. Although our studies do not address the question of whether PTEN has any
protein phosphotyrosyl substrates, it seems unlikely that such substrates would show significant rates of dephosphorylation when compared with PtdIns(3,4,5)P₃, given the large discrepancy between the latter and even a closely similar, soluble substrate such as Ins(1,3,4,5)P₆. Nevertheless it would be valuable to identify a mutation in PTEN that prevented tyrosine phosphatase activity without affecting the lipid phosphatase, since such a reagent would have the potential to reveal any PTEN functions that are independent of lipid phosphatase activity.

Unlike many second messenger-metabolizing enzymes, PTEN appears to be constitutively active, thereby making it well suited to maintaining low basal levels of PtdIns(3,4,5)P₃, as well as being capable of blunting agonist-stimulated increases in this signal. This property is clearly an important feature of the tumour suppressor activity of PTEN. Moreover, it is not obvious that PTEN is regulated through acute covalent modifications or by translocation to signalling complexes. Changes in the level of expression of PTEN caused by stimuli such as transforming growth factor β or pharmacological agents such as PPARγ agonists (or in heterozygotes with only one functional PTEN allele) appear to influence directly the set point of PI 3-kinase signalling activity. A key next step will be to identify the PTEN promoter in order to explore in more detail mechanisms of transcriptional regulation of enzyme expression.

References