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Expanding coincident signaling by PTEN through its inositol 1,3,4,5,6-pentakisphosphate 3-phosphatase activity

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Abstract PTEN, a tumor suppressor among the most commonly mutated proteins in human cancer, is recognized to be both a protein phosphatase and a phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) 3-phosphatase. Previous work [Maehama and Dixon, J. Biol. Chem. 273 (1998) 13375–13378] has led to a consensus that inositol phosphates are not physiologically relevant substrates for PTEN. In contrast, we demonstrate that PTEN is an active inositol 1,3,4,5,6-pentakisphosphate (Ins(1,3,4,5,6)P_5) 3-phosphatase when expressed and purified from bacteria or HEK cells. Kinetic data indicate Ins(1,3,4,5,6)P_5 (K_m = 7.1 μM) and PtdIns(3,4,5)P_3 (K_m = 26 μM) compete for PTEN in vivo. Transient transfection of HEK cells with PTEN decreased Ins(1,3,4,5,6)P_5 levels. We discuss the physiological significance of these studies in relation to recent work showing that dephosphorylation of Ins(1,3,4,5,6)P_5 to inositol 1,4,5,6-tetrakisphosphate is a cell signaling event.

Key words: PTEN; Phosphatidylinositol 3,4,5-trisphosphate; Inositol 1,3,4,5,6-pentakisphosphate; 3-Phosphatase; Inositol phosphate

1. Introduction

Signal transduction modules receive and transmit intracellular information. Some proteins integrate multiple inputs into a single output. Other proteins display 'coincident signaling', by simultaneously regulating divergent signaling pathways. Together these processes provide signaling specificity and regulate 'cross-talk'; a challenge for signal transduction research is to unravel the molecular processes involved.

Coincident signaling is undertaken by PTEN, a tumor suppressor that ranks among the most commonly mutated proteins in human cancer [1]. PTEN is both a protein phosphatase [2] and a phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) 3-phosphatase [3]. Dephosphorylation of focal adhesion kinase by PTEN inhibits cellular growth, invasion, migration and focal adhesions [4]. PtdIns(3,4,5)P_3 phosphatase activity of PTEN decreases cell proliferation and antagonizes Akt-dependent cell survival [1]. However, in several earlier studies, PTEN overexpression in cells was found to have divergent effects that could, for example, range from cell cycle arrest to apoptosis, and it has been difficult to reconcile all of these effects solely from PTEN's inositol lipid and protein phosphatase activities [1]. Thus, evaluating if PTEN might utilize further substrates has been promoted as an important challenge for future work with this protein [1].

A previous study concluded that PTEN showed negligible activity towards the inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) head group of PtdIns(3,4,5)P_3 (K_m = 99 μM [3]). Thus, there has not been any prior interest in the possibility that PTEN might hydrolyze inositol phosphates. Indeed, it is generally accepted that inositol phosphates and inositol lipids are each predominantly metabolized by their own dedicated kinases and phosphatases with no substantial cross-over in substrate specificity. For example, the type I and II '3-phosphatases individually prefer either inositol 1,3-bisphosphate (Ins(1,3)P_2) or phosphatidylinositol 3-monophosphate respectively [5]. The inositol lipid 4-phosphatases have only relatively weak activities against Ins(1,3,4)P_3 and Ins(3,4)P_2 [6]. The type 1 5-phosphatase is specialized for hydrolyzing Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, and has little activity against the inositol lipids [7]. The other 5-phosphatases have high activities against PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 [7], and preferential hydrolysis of the lipids in vivo is also favored by their being more abundant than Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 [8]. Thus, there is the firm belief that any metabolic competition between inositol lipids and inositol phosphates is of minimal regulatory significance in vivo.

In this report, using structural, molecular and biochemical approaches, we expand PTEN's signaling repertoire by showing it actively hydrolyzes inositol pentakisphosphate (InsP_5) to Ins(1,4,5,6)P_4. This catalytic reaction generates an antagonist of PtdIns(3,4,5)P_3 [9], and a putative transcriptional regulator [10], and may influence Rho-GTPase activation [11]. This metabolic competition between an inositol lipid and an inositol phosphate adds a new, distinctive property to PTEN functionality.

2. Materials and methods

2.1. Materials

[^{3}H]Ins(1,3,4,5,6)P_5 was prepared as previously described [12]. ^{14}C-labeled Ins(1,3,4,5)P_4, Ins(1,3,4,6)P_4 and D/L-Ins(1,4,5,6)P_4 were isolated from [^{14}C]inositol-labeled, parotid acinar glands [13]. Non-radioactive Ins(1,3,4,5,6)P_5 was supplied by CellSignals (Lexington, KY, USA), and inositol hexakisphosphate (InsP_6) was purchased from Calbiochem (La Jolla, CA, USA). C_4PtdIns(3,4,5)P_3 was purchased from Echelon (Salt Lake City, UT, USA). GST-PTEN was

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expressed in *Escherichia coli*, purified as previously described [3], and kindly provided to us by Maehama and Dixon. The PTEN was stored in aliquots at −70°C. Earlier work also describes the DNA constructs encoding FLAG-tagged PTEN under the control of the CMV promoter in vector pCMV5 [3]. Recombinant human Ins(3,4,5,6)P_4 1-kinase was prepared as described previously [14]. The yeast protein Arg-82, which shows Ins(1,4,5,6)P_4 3-kinase activity, was prepared as previously described [15].

2.2. PTEN transfection and inositol phosphate analysis of HEK293 cells

HEK293 cells (2×10^5 cells/well) were seeded in 6 well plates in DMEM/10% fetal bovine serum and grown overnight at 37°C in 5% CO_2. Cells were radiolabeled with 25 μCi/ml [^{3}H]inositol (ARC, St. Louis, MO, USA) for 3–4 days, and then each well was transfected with 5 μg plasmid DNA encoding PTEN or vector control using Lipofectamine 2000 transfection reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's recommended protocol. After a further 4–6 h, lipid–DNA complexes were removed and replaced with fresh complete medium, including [^{3}H]inositol, for an additional 36–48 h. Cells were quenched with 1 ml 2 M perchloric acid supplemented with 0.1 mg/ml InsP_6 and 2 mM EDTA. Lysates were neutralized and analyzed by HPLC on a Partisphere SAX column as previously described [15].

2.3. Western blotting and detection of FLAG-tagged proteins

Unlabeled HEK293 cells were grown and transfected as described above. Cells were harvested by trypsinization, washed twice with phosphate-buffered saline (PBS), and frozen at −30°C. Cell pellets were subsequently thawed and lysed in 0.1 ml protein extraction buffer (50 mM β-glycerophosphate, pH 8.2, 250 mM sucrose, 150 mM NaCl, 4 mM CHAPS, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 μM AEBSF, 10 μM E-64, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). FLAG-tagged PTEN was detected using the anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, MO, USA), followed by a horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Amersham Pharmacia, Piscataway, NJ, USA). Protein concentration was determined by Bradford analysis using bovine serum albumin (BSA) as standard, and 25 μg aliquots of cell extracts were subjected to Western blotting from 10% polyacrylamide NuPAGE/Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) onto nitrocellulose membranes.

2.4. FLAG-PTEN purification

HEK293 cells were transfected with wild type (WT) or C124S mutant FLAG-PTEN as described above. After 36–48 h, cells were trypsinized, washed twice in PBS, and harvested in 0.5 ml protein extraction buffer (see above), but lacking NaF. Extracts were cleared by centrifugation, and supernatants were incubated with 0.1 ml anti-FLAG M2 agarose (Sigma) for 1–2 h at 4°C with gentle rocking. The beads were recovered by centrifugation, washed three times in extraction buffer, and PTEN were released in 0.1 ml 0.5 M FLAG peptide in Tris-buffered saline (TBS) on ice for 30 min. Affinity purified proteins were separated by SDS–PAGE on 10% polyacrylamide NuPAGE Bis-Tris gels and detected by silver staining. Protein concentration was determined by densitometry of stained gels using known amounts of purified BSA (Pierce, Rockford, IL, USA) as a standard.

2.5. Assays of inositol phosphate metabolism

PTEN was assayed in buffer comprising 50 mM HEPES (pH 7.2), 10 mM DTT, 0.1 mg/ml BSA. Samples were acid quenched, neutralized and analyzed either by gravity-fed anion exchange columns [16] or by HPLC [15].

Table 1
Kinetic parameters for PTEN

Substrate	K_m (μM)	V_{max} (nmol/mg/min)
C_4PtdIns(3,4,5)P_3	26	60
Ins(1,3,4)P_3	11.5	24
Ins(1,3,4,5)P_4	4.6	112
InsP_5	7.1	67

Assay details are given in Section 2, and data represent mean values from two to three experiments.

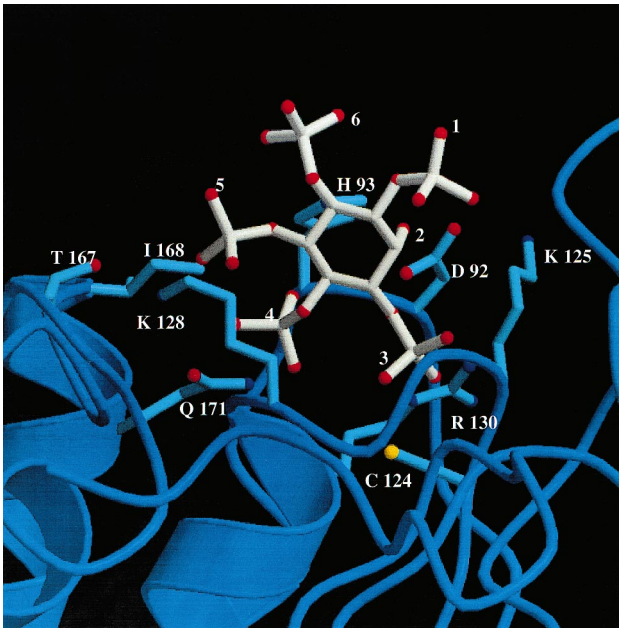

Fig. 1. Molecular model of InsP_5 accessing the active site of PTEN. Using the modeling approach taken previously for Ins(1,3,4,5)P_4 [17], we docked Ins(1,3,4,5,6)P_5 (modeled using InsP_6 from protein data bank entry 1DKP) into the active site of PTEN (protein data bank entry 1D5R). The additional 6-phosphate of InsP_5 is easily accommodated without steric conflicts. However, while the 2-hydroxyl group can interact favorably with Asp-92, a 2-phosphate (as in InsP_6) clashes sterically with Asp-92 and would also be electrostatically repelled.

3. Results and discussion

3.1. Hydrolysis of Ins(1,3,4,5)P_4 by PTEN

PTEN is an active PtdIns(3,4,5)P_3 3-phosphatase [3]. Our determination of the V_{max} for the substrate PtdIns(3,4,5)P_3 (Table 1), is within the range (20–200 nmol/mg/min) typically quoted for PTEN [17,18]. Analysis of the enzyme crystal structure has previously predicted that the active site can accommodate Ins(1,3,4,5)P_4 [17], which represents the polar head group of PtdIns(3,4,5)P_3. However, a different study indicated that the K_m value for Ins(1,3,4,5)P_4 is 99 μM, and the V_{max} value is only 8.5 nmol/mg/min [3]. The substrate affinity and V_{max} value is much larger for PtdIns(3,4,5)P_3 (Table 1). Thus, there has not previously been any serious consideration that an inositol phosphate might be a physiologically relevant substrate for PTEN.

Surprisingly, we found that the affinity of recombinant PTEN for Ins(1,3,4,5)P_4 ($K_m = 4.6$ μM, Table 1) is >20-fold higher than the value previously determined by Maehama and Dixon [3]. Moreover, our V_{max} value for Ins(1,3,4,5)P_4 (112 nmol/mg/min, Table 1) is 13-fold larger than previously determined [3]. We have not studied why our kinetic parameters are so different from the earlier report, but it is important to note that these preparations of PTEN were kindly provided to us by Maehama and Dixon. Nevertheless, at levels of 0.1–1 μM in vivo, Ins(1,3,4,5)P_4 would not be expected to appreciably compete with PtdIns(3,4,5)P_3, which may reach 200 μM during cell activation [8]. However, our data prompted us to study whether other inositol phosphates might be more relevant substrates for PTEN.

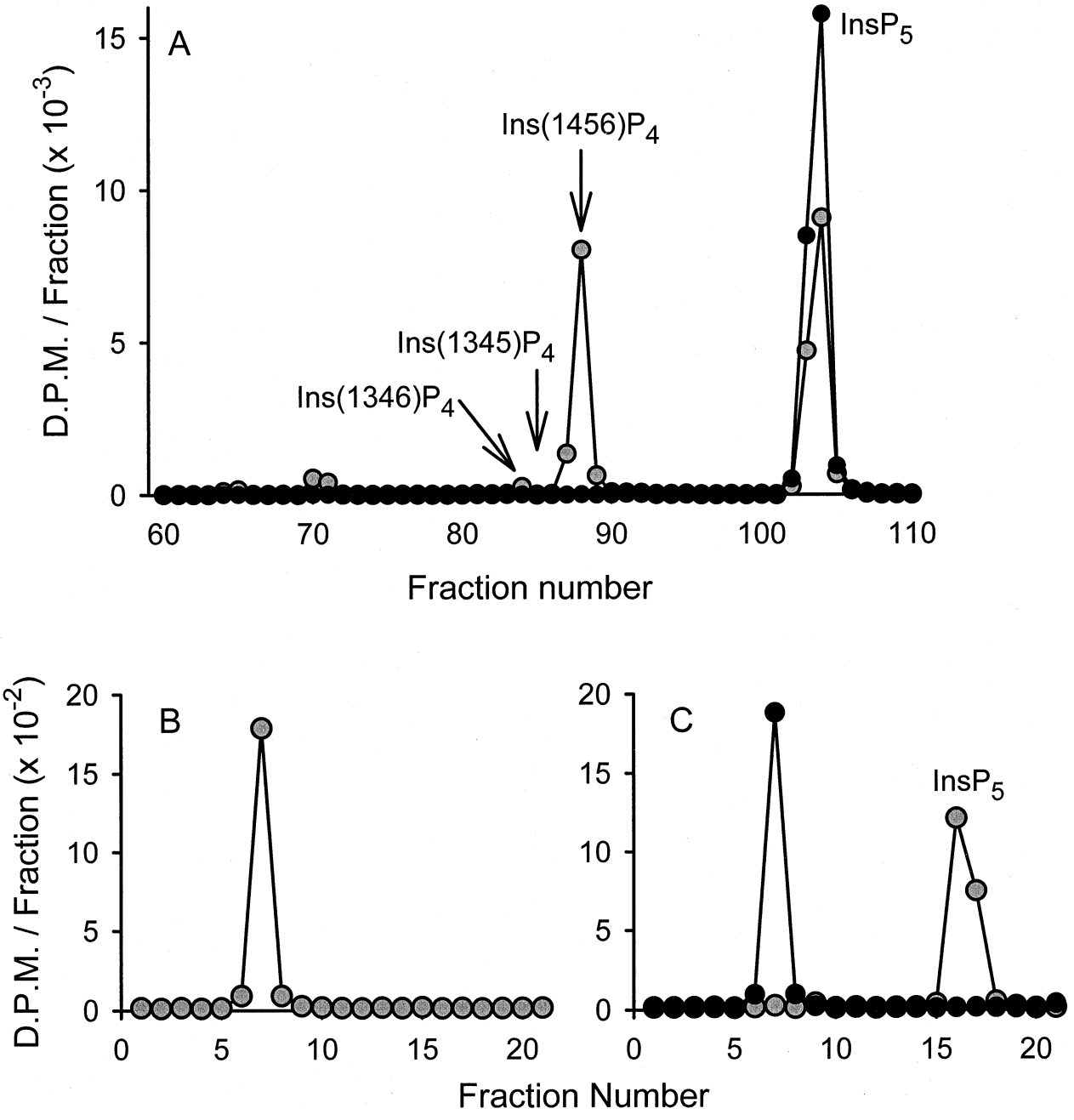

Fig. 2. HPLC analysis of PTEN activity towards $InsP_5$. A: PTEN (34 μg) was incubated for 0 min (black circles) or 20 min (gray circles) at 37 °C in 200 μl assay buffer (Table 1) containing 50 μM [^{3}H]$InsP_5$. Reactions were analyzed by HPLC using a Synchropak Q100 column. Arrows mark the elution positions of ^{14}C-labeled internal standards of Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4 and a Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 mixture. The PTEN-generated [^{3}H]$InsP_4$ product was further analyzed stereospecifically; approx. 2000 dpm was incubated for 0 min (black circles) or overnight (gray circles) with either (B) recombinant Ins(3,4,5,6)P_4 1-kinase [14] or (C) recombinant Ins(1,4,5,6)P_4 3-kinase (Arg-82); $InsP_5$ accumulation was measured by HPLC using a Partisphere SAX column.

3.2. *Hydrolysis of Ins(1,3,4,5,6)P_5 by PTEN*

We used molecular modeling techniques to predict that the active site of PTEN could accommodate $InsP_5$ (Fig. 1). No steric conflicts are predicted. In particular, the additional, bulky 6-phosphate group (which is absent in Ins(1,3,4,5)P_4 and PtdIns(3,4,5)P_3) is predicted to be orientated away from the surface of the protein (Fig. 1). This observation provides an unexpected potential rationale for the large size of the active site cleft in PTEN [17]. We next empirically verified that recombinant PTEN dephosphorylated $InsP_5$ (Table 1, Fig. 2). The value of the K_m was 7.1 μM, well below the generally accepted physiological levels of this particular substrate (15–50 μM [19,20]). In fact, PTEN has higher affinity for $InsP_5$ than it does for PtdIns(3,4,5)P_3, while the V_{max} values for both substrates are comparable (Table 1). Thus, $InsP_5$ is likely to be just as physiologically relevant a substrate for PTEN as is PtdIns(3,4,5)P_3, which provides the most notable exception to the general rule that there is little metabolic competition between inositol phosphates and inositol lipids (see Section 1).

The signaling significance of PTEN-catalyzed $InsP_5$ hydrolysis depends upon the reaction specificity. HPLC analysis indicated $InsP_5$ was dephosphorylated to a single $InsP_4$ peak that co-eluted with a standard Ins(1,4,5,6)P_4/Ins(3,4,5,6)P_4 mixture, which in turn was resolved from Ins(1,3,4,6)P_4, Ins(1,3,4,5)P_4, and the latter's stereoisomer, Ins(1,3,5,6)P_4 (Fig. 2A). This leaves Ins(3,4,5,6)P_4 and Ins(1,4,5,6)P_4 as the only possible constituents of the $InsP_4$ peak. However, further enzymatic analysis detected only Ins(1,4,5,6)P_4 (Fig. 2B,C). Thus, PTEN specifically removes the 3-phosphate from $InsP_5$, forming Ins(1,4,5,6)P_4. The high selectivity of PTEN for the 3-phosphate of PtdIns(3,4,5)P_3 and $InsP_5$ is reinforced by its inability to hydrolyze either Ins(1,4)P_2 or Ins(1,4,5)P_3 (data not shown, and [3]). Ins(1,3,4)P_3 (K_m = 11.5 μM, Table 1) was a relatively weak substrate. $InsP_6$ was not attacked by PTEN, even when it was incubated at 20 times the level of enzyme that completely dephosphorylated $InsP_5$ (data not shown). Indeed, our modeling analysis predicts that $InsP_6$ is prevented from accessing the active site by its 2-phosphate (Fig. 1, legend). Thus, of all the inositol phosphates that we have examined, Ins(1,3,4,5,6)P_5 is the only one with any physiological relevance as a substrate for PTEN.

The aforementioned experiments used bacterially expressed PTEN. We next checked the catalytic activity towards $InsP_5$ of FLAG-tagged PTEN which was expressed in human HEK293 cells. The PTEN was affinity purified using an immobilized anti-FLAG antibody (Fig. 3, lane 1). 10 μM [^{3}H]$InsP_5$ was hydrolyzed at a rate of 22 ± 7 nmol/mg/min (n = 4), close to the approx. 35 nmol/mg/min of the bacterially expressed enzyme (see Table 1). There was no detectable activity in immunoprecipitates prepared from cells transfected with vector alone. A C124S PTEN mutant (Fig. 3, lane 2), known not to hydrolyze PtdIns(3,4,5)P_3 or phosphoprotein [3,17], was also inactive against $InsP_5$ (< 1 nmol/mg/min). The latter result confirms that $InsP_5$ competes with both PtdIns(3,4,5)P_3 and phosphoproteins for the same active site of PTEN. Occasions when this might have regulatory significance are during cell cycle progression [21], or upon cellular differentiation [22], whereupon regulated fluctuations in Ins(1,3,4,5,6)P_5 levels would seem likely to impact PTEN activity towards its alternative substrates.

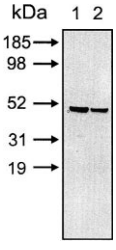

Fig. 3. Purification of recombinant PTEN from HEK293 cells. HEK293 cells were transfected (Lipofectamine 2000) with 5 μg plasmid DNA encoding either WT or C124S mutant FLAG-PTEN in vector pCMV5. After 36–48 h, cells were lysed in 50 mM β-glycerophosphate, pH 8.2, 250 mM sucrose, 150 mM NaCl, 4 mM CHAPS, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 μM AEBSF, 10 μM E-64, 1 μg/ml leupeptin, 1 μg/ml pepstatin A. PTEN was bound to anti-FLAG M2-agarose (Sigma), washed with extraction buffer, and released with 0.5 M FLAG peptide in TBS. Aliquots (10 μl) of WT (lane 1) and C124S (lane 2) FLAG-PTEN were separated by SDS–PAGE on 10% polyacrylamide NuPAGE Bis-Tris gels and silver stained.

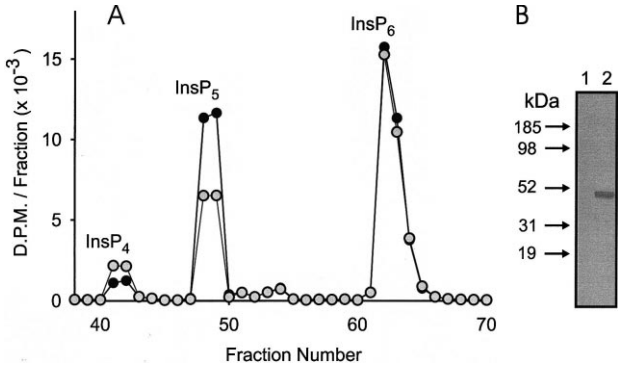

Fig. 4. Effect of PTEN upon [^{3}H]$InsP_5$ levels in HEK293 cells. A: HEK293 cells were labeled with 25 μCi/ml [^{3}H]inositol (ARC, St. Louis, MO, USA) for 3–4 days, and then transfected (see Fig. 3, legend) with either FLAG-PTEN (gray circles) or vector alone (black circles). After 4–6 h, medium was replaced with fresh [^{3}H]inositol-supplemented medium for an additional 36–48 h. Cells were quenched and analyzed by HPLC using a Synchropak Q100 column. Peaks were identified by comparison with the appropriate standards. Data are representative of seven experiments (see text). B: Anti-FLAG M2 monoclonal antibody (Sigma), followed by an HRP-conjugated anti-mouse IgG antibody, was used for Western blots of 25 μg lysate, from vector-transfected (lane 1) or FLAG-PTEN-transfected (lane 2) cells (see Fig. 3, legend).

Moreover, the dephosphorylation of $InsP_5$ to Ins(1,4,5,6)P_4 itself has a number of important signaling consequences. First, the PtdIns(3,4,5)P_3 signaling pathway is antagonized by Ins(1,4,5,6)P_4, possibly because the latter competes for certain PtdIns(3,4,5)P_3 binding domains [9]. This suggests PTEN undertakes bimodal deactivation of PtdIns(3,4,5)P_3 signaling, since the accumulation of Ins(1,4,5,6)P_4 is a negative signal that reinforces the consequences of dephosphorylation of PtdIns(3,4,5)P_3 itself. Second, Ins(1,4,5,6)P_4 has been proposed to regulate transcription, at least in yeast [10]; interestingly, some mammalian PTEN is nuclear [23]. Third, the dephosphorylation of $InsP_5$ to Ins(1,4,5,6)P_4 is closely associated with Rho-GTPase activation [11].

3.3. PTEN is a unique Ins(1,3,4,5,6)P_5 phosphatase

The only other known mammalian $InsP_5$ 3-phosphatase is 'multiple inositol polyphosphate phosphatase' (MIPP); however, the latter is closeted inside endoplasmic reticulum with only restricted access to inositol phosphates [16]. The cytosolic Ins(1,4,5,6)P_4 3-kinase [20] was previously enigmatic in the absence of a satisfactory explanation for an active pathway of Ins(1,4,5,6)P_4 synthesis. Now, however, we can appreciate the occurrence of an ongoing Ins(1,3,4,5,6)P_5 3-phosphatase/Ins(1,4,5,6)P_4 3-kinase substrate cycle in mammalian cytosol. Perturbations in this cycle's dynamic equilibrium are deleterious to cell function [16], and could be effected upon relocation of PTEN from the nucleus to the cytoplasm, as occurs during neoplastic transformation of certain tumors [23].

There is a Mg^{2+}-independent, $C(X)_5R(S/T)$ phosphatase consensus within the active site of PTEN. A virulence protein in *Salmonella*, SopB, which cleaves the 3-phosphate from Ins(1,3,4,5,6)P_5 [9,11], also has $C(X)_5R(S/T)$ at the core of its active site, but inositol lipids are hydrolyzed only weakly, and with a different specificity (4-phosphatase) [24]. There are also dedicated inositol lipid 4-phosphatases with the $C(X)_5R(S/T)$ sequence [6]. Sac domains, which hydrolyze PtdIns4P and some other inositol lipids, incorporate the

$C(X)_5R(S/T)$ motif [25]. We immunoprecipitated synaptojanin from a rat brain extract [26] and assessed the catalytic activity of its Sac domain as detailed in the Table 1 legend. The rate of PtdIns4P hydrolysis was 4 pmol/min/μl (no activity was observed using preimmune sera). However, immunoprecipitates of synaptojanin did not hydrolyze $InsP_5$ (data not shown). Thus, the catalytic site of PTEN is empowered with unique coincident signaling properties.

3.4. Hydrolysis of Ins(1,3,4,5,6)P_5 by PTEN in vivo

When PTEN was transfected into cells in earlier studies, it had been assumed that any physiological effects were due to the protein's PtdIns(3,4,5)P_3 phosphatase and/or protein phosphatase activities. In view of our discovery that PTEN is an Ins(1,3,4,5,6)P_5 3-phosphatase, we next determined if enhanced $InsP_5$ hydrolysis is observed upon ectopic PTEN expression. We transiently transfected HEK293 cells with FLAG-tagged PTEN, because some cell types cannot tolerate long-term increases in cytosolic $InsP_5$ phosphatase activity (at least following overexpression of an aberrant MIPP lacking its N-terminal endoplasmic reticulum-targeting sequence [16]). A 50 kDa FLAG-tagged protein was observed in PTEN-transfected cells (Fig. 4B, lane 2, predicted size 47.4 kDa), but not in vector-transfected controls (Fig. 4B, lane 1). HPLC (Fig. 4A) was used to assess $InsP_5$ levels (which were normalized to $InsP_6$, which was unaffected by PTEN transfection: 34473 ± 8879 dpm/sample in vector-transfected cells vs. 34173 ± 7464 dpm/sample in PTEN-transfected cells, $n = 7$). In control cells, the ratio of $InsP_5/InsP_6$ was 0.71 ± 0.05 ($n = 7$). The corresponding ratio for PTEN-transfected cells was 0.54 ± 0.05 ($n = 7$). This represents a significant decrease of 25%. The degree of this effect is remarkably close to the 30% decline in PtdIns(3,4,5)P_3 levels previously observed following transient PTEN overexpression in HEK cells [2]. PTEN slightly increased levels of $InsP_4$ (0.088 ± 0.01 in vector-transfected cells; 0.12 ± 0.02 in PTEN-transfected cells) but probably dephosphorylation of this $InsP_4$ [9] prevented it from accumulating further. Our data confirm that PTEN does dephosphorylate $InsP_5$ in vivo; this may contribute to some of the phenotypic effects that accompany PTEN overexpression. A new dimension to future PTEN research will be to study whether perturbations in the $InsP_5$/Ins(1,4,5,6)P_4 cycle also contribute to the neoplastic consequences of *PTEN* gene mutations.

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