We show that a novel PTEN-like phosphatase (PLIP) exhibits a unique preference for phosphatidylinositol 5-phosphate (PI(5)P) as a substrate in vitro. PI(5)P is the least characterized member of the phosphoinositide (PI) family of lipid signaling molecules. Recent studies suggest a role for PI(5)P in a variety of cellular events, such as tumor suppression, and in response to bacterial invasion. Determining the means by which PI(5)P levels are regulated is therefore key to understanding these cellular processes. PI(5)P is highly enriched in testis tissue and, similar to other PI phosphatases, exhibits poor activity against several proteinaceous substrates. Despite a recent report suggesting a role for PI(5)P in the regulation of Akt, the overexpression of wild-type or catalytically inactive PLIP in Chinese hamster ovary-insulin receptor cells or a dsRNA-mediated knockdown of PLIP mRNA levels in Drosophila S2 cells does not alter Akt activity or phosphorylation. The unique in vitro catalytic activity and detailed biochemical and kinetic analyses reported here will be of great value in our continued efforts to identify in vivo substrate(s) for this highly conserved phosphatase.

Protein-tyrosine phosphatases (PTPs) are a family of ~100 phosphatases characterized by their highly conserved C$_X$R catalytic motif. Once thought to dephosphorylate only phosphotyrosine residues, a subset of PTPs are now known to dephosphorylate phosphoserine- and phosphothreonine-containing proteins, as well as to use RNA and phosphoinositides as substrates. PI(5)P is the least characterized PI, having only recently been identified as an endogenous lipid (16). Recent studies (7, 17, 18) report changes in intracellular PI(5)P levels during cell cycle progression, as well as upon thrombin treatment and osmotic stress. Furthermore, the plant homedomain-containing ING2 protein, a candidate tumor suppressor, was recently shown to act as a nuclear PI(5)P receptor, a function that regulates its ability to activate p53 (19). PI(5)P has also been tied to tumor suppression via its potential regulation of Akt. It was recently demonstrated that the loss of PI(5)P, via a conversion to PI(4,5)P$_2$ by the phosphoinositide kinase PIP4K II, resulted in a decrease in Akt activity (20). Lastly, PI(5)P has been shown to enhance the activity of various myotubularin phosphatases (MTM1, MTMR3, and MTMR6) toward their preferred substrate PI(3,5)P$_2$ presumably through allosteric regulation (21). Together these studies stress the importance of PI(5)P as a bona fide signaling molecule, and not merely a metabolic precursor to other PIs as once proposed.

Here, we report the first example of a mammalian phosphatase utilizing PI(5)P as its preferred substrate. PLIP exhibits a highly selective in vitro activity against PI(5)P at a level comparable with that of PTEN against its preferred substrate, PI(3,4,5)P$_3$. To date, we have not demonstrated the ability of PLIP to alter PI(5)P levels in vivo. Thus, despite having poor activity toward several proteinaceous substrates, PLIP may still possess a highly specific proteinaceous substrate not assessed in this study. The continued analysis of endogenous...
Fig. 1. PLIP primary sequence analysis. A, primary sequence alignment of the P-loop regions of PTPs possessing a PTEN-like catalytic motif. Catalytic cysteine and arginine residues are highlighted in red, and active site basic residues are in blue. The phosphate position of the inositol ring dephosphorylated by each enzyme is indicated on the right. B, primary sequence alignment of the P-loop region of various PLIP orthologs organized by ClustalW alignment. The cladogram at the left shows the evolutionary progression of PLIP orthologs through four kingdoms (Eubacteria, Pirellula sp.; Protista, D. discoideum; Plantae, P. taeda through H. magnipapillata; Animalia, E. granulosus through H. sapiens). "PROT" denotes sequences derived from characterized proteins or predicted proteins from fully sequenced genomes, "EST" denotes sequences derived from expressed sequence tags. NCBI accession numbers are given at right. (Note: cladogram not to scale.) C, primary sequence of murine PLIP. Shaded region, beginning with green-boxed-start methionine, indicates the sequence of the clone used in this study. The catalytic P-loop region is highlighted in red.
substrates for PLIP should be greatly aided by the extensive biochemical characterization of this enzyme and its highly specific activity against PI(5)P.

EXPERIMENTAL PROCEDURES

Expression and Purification of PLIP—Recombinant murine PLIP was expressed as a fusion protein with N-terminal GST and His6 tags in Escherichia coli BL21 (DE3) CodonPlus RIL cells (Stratagene). The expression construct was assembled by ligating a PCR product encoding full-length PLIP into the 5′/HincII and 3′/EcoRI sites of a modified pET41 vector (pSJ6), kindly provided by Zhaohui Xu (University of Michigan, Ann Arbor, MI). One-liter bacterial cultures were grown at 37 °C in 2× YT medium (8 g of tryptone, 5 g of yeast, 5 g of NaCl/liter) possessing chloramphenicol (34 μg/ml) and kanamycin (50 μg/ml) to an optical density of 0.5–0.7. Cultures were then chilled on ice for 20 min, supplied with fresh antibiotics, induced with isopropyl-1-thio-D-galactopyranoside to a final concentration of 0.4 mM, and allowed to grow overnight at 25 °C as described previously (22). All subsequent steps were performed at 4 °C. The cultures were pelleted by spinning at 6000 rpm for 10 min in a Sorvall GSA rotor, resuspended in 30 ml of buffer (20 mM imidazole, 50 mM Tris, pH 7.4, 300 mM NaCl) with complete protease inhibitors (Roche Applied Science), and lysed via French press.

![Graphs and Tables]

**Fig. 2. Analysis of PLIP activity against phosphoinositides.** A, wild-type (WT) or catalytically inactive (C132S) PLIP was tested against a panel of water-soluble BODIPY-tagged di-C6 phosphoinositides. Conversion of PI(5)P to PI by wild-type PLIP is indicated with a red arrow. Migration distances of the various PI derivatives are indicated on the right. B and C, activity of PLIP or PTEN against di-C8 PIs incorporated into a lipid bilayer with phosphatidylserine carrier lipid. D, activity of PLIP against water-soluble PI(5)P derivatives of varying acyl chain length.

| Substrate       | Conc. (μM) | pmol/min·μg | Error (+/−) |
|-----------------|------------|-------------|-------------|
| Inositol (1,5)P2 | 50         | 17          | 4           |
| di-C4 PI(5)P    | 50         | 252         | 28          |
| di-C8 PI(5)P    | 50         | 2189        | 231         |
Insoluble material was removed by centrifugation at 50,000 rpm for 30 min. The fusion protein was purified from the soluble supernatant first over Ni²⁺-agarose beads and then by successive saturation on Superdex 75 and 260 gel filtration columns (Amersham Biosciences). The resulting protein was concentrated, flash frozen in liquid nitrogen, and stored at −80 °C in 25% glycerol, 2 mM EDTA, and 2 mM dithiothreitol until use.

**Fluorescent Phosphoinositide Assays**—Fluorescent PI, TIC, and malachite green assays were performed as described recently (22). Eighteen microliters of assay buffer (50 mM ammonium acetate, pH 5.5, 0.1% (v:v) 2-mercaptoethanol (Sigma)) containing 1 μg of a Di-C₅₄-NBD phosphoinositide (Echelon) were prewarmed at 37 °C for 5 min. Reactions were initiated by the addition of 2 μl of 0.1 μg/μl GST-PLIP diluted in assay buffer containing 1.0 mg/ml gelatin. Assays were quenched after 2.0 min by the addition of 100 μl of acetonitrile and were dried in a Speed Vac at medium heat.

**Malachite Green Assays**—Di-C₅₄ phosphoinositides and dioleoyl-phosphatidylserine (Sigma) were dried together in a Speed Vac and resuspended via sonication in 18 μl of assay buffer (100 μM sodium acetate, 50 μM bis-Tris, 50 μM Tris, pH 5.5, 10 mM dithiothreitol) to final concentrations of 50 and 500 μM, respectively. After prewarming at 37 °C for 5 min, reactions were initiated by the addition of 20 ng of Malachite Green reagent and vortexed. Samples were allowed to sit for 40 min for color development before measuring absorbance at 705/50 nm.

**Phosphatase Assays**—Phosphatase activities were measured in mole of phosphate released/min-mol of enzyme. All measurements were performed in triplicate. S.E. was less than 5% in all cases.

**Table I**

Phosphatase activity of various PTPs

|                | TC-PTP | VHR | PLIP | PTEN | MTM1 |
|----------------|--------|-----|------|------|------|
| Pl (3) P       | ND     | -0.06 | -0.06 | 135.9 | 20.4 |
| Pl (5) P       | -0.06  | -0.06 | 151.9 | 23.7 |
| Pl (3, 4, 5) P | ND     | ND   | 3.8   | 27.7 | 76.9 |
| MyBP Tyr(P)    | 195.9  | 0.55 | 0.1   | 0.73 | 0.06 |
| Casein Ser(P)  | 1440.0 | 1.88 | 0.2   | 0.03 | 0.1 |
| MyBP Ser/Thr(P) | -0.06 | -0.06 | -0.06 | -0.06 | -0.06 |
| Casein Ser(P)  | -0.06  | -0.06 | -0.06 | -0.06 | -0.06 |

a ND, not determined.

b This study.

c Ref. 43.
d Ref. 32.

e ND, not determined.

**RESULTS AND DISCUSSION**

**Identification of PLIP as a PTEN-like Phosphatase**—Elucidation of the crystal structure of PTEN revealed the importance of the conserved active site lysine residues (Lys-125, 129, 130) in establishing a negatively charged catalytic pocket conducive to the binding of its preferred substrate, PI(3,4,5)P₃ (6). In an attempt to identify additional phosphatases possessing this motif, we conducted PSI-BLAST analyses using the PTEN phosphatase domain as a query (26, 27). We subsequently identified a predicted protein (NCBI accession, XP_374879) that possessed the PTEN-like active site CKAGRSR. PTENs from all sources have basic amino acids in the highlighted positions. Using the murine ortholog of this protein, we conducted multiple PSI-BLAST and TBLASTN searches against the non-redundant and EST databases. Through this analysis, we identified what we believe are over 60 orthologs of a protein we now call PLIP. Included in these results is an ortholog from the eubacterium *Pirellula* sp. strain 1 whose defining characteristics include an intracellular membrane and various eukaryotic-like lipids (28, 29). The active site region of PLIP shows remarkable evolutionary conservation, exhibiting greater than 70% identity/80% similarity in orthologs from four different phylogenetic kingdoms (Fig. 1B). Such conservation supports the notion that residues within this region are critical for proper enzyme function, most likely in establishing substrate specificity.

**PLIP Exhibits PI(5)P Phosphatase Activity**—The murine ortholog of PLIP (Fig. 1C), was cloned into a GST bacterial expression vector, expressed, and purified to near homogeneity. The predicted amino acid sequence of this ortholog (NCBI accession number gi:23956130) has an extended N terminus (Fig. 1C, residues 1–69). However, as this region was not found in any of the more than 60 other PLIP orthologs, it was omitted from the final GST-PLIP construct used for the biochemical characterization of PLIP.

A pH rate profile for PLIP performed with various substrates revealed this enzyme to be most efficient at pH 5.5 (data not shown). Therefore, all further assays were performed at this pH. Because PLIP had active site residues similar to those found in PTEN, we examined its activity toward a panel of fluorescently labeled di-C₅₄ phosphoinositides. A TLC analysis of the reaction products, shown in Fig. 2A, revealed that PLIP exhibits a highly selective substrate specificity for PI(5)P. This substrate preference is shared by the *Dictyostelium* ortholog of PLIP (30). As expected, the mutation of the predicted catalytic cysteine residue to serine (C132S) nullified this activity. To further confirm this result, PLIP was tested against a panel of di-C₅₄ PIs presented in a lipid bilayer with phosphatidylserine.
carrier lipid. PLIP again demonstrated robust activity against PI(5)P, 44-fold greater than against PI(3,5)P2, its second most preferred substrate (Fig. 2B). This is a notable enhancement in specificity compared with PTEN, which exhibits only a 1.8-fold preference for PI(3,4,5)P3 over PI(3,5)P2 (Fig. 2, B and C). PLIP assayed against di-C16 PIs revealed highly similar results (data not shown).

We also tested water-soluble PI(5)P of multiple acyl chain lengths as substrates for PLIP, as this variable has been shown to affect the level of enzymatic activity for other PI phosphatases (31, 32). PLIP demonstrated a 10-fold increase in activity against di-C4 PI(5)P versus the inositol head group, inositol (1,5)P2, and a second 10-fold increase against di-C8 PI(5)P versus di-C4 PI(5)P (Fig. 2D) suggesting that this lipid moiety is also an important factor for PLIP activity.

**PI(5)P Activity Is Not a General Feature of PTPs**—Because of the only recent emergence of PI(5)P as a known signaling molecule, few PTPs have been tested for activity against this substrate. To ensure that activity against PI(5)P is not a common feature of PTPs, membrane-bound di-C8 PI(5)P was assayed using a known tyrosine-specific PTP (T-cell-PTP) and a known dual-specific PTP (VH-1-related) (33–37). Both enzymes yielded activity that was barely detectable above background indicating that PI(5)P activity is not a general feature of the various classes of PTPs (Table I). PTEN and MTM1, known PI phosphatases, both possess the ability to dephosphorylate PI(5)P although with 17- and 200-fold less efficiency than toward their preferred substrates, respectively.

**PLIP Exhibits Poor Protein Phosphatase Activity**—A characteristic of all PI phosphatases to date has been their extremely poor activity toward proteinaceous substrates (32). To determine whether PLIP is consistent with this trend, its activity was measured against the artificial phosphotyrosine analog, pNPP, along side with di-C8 PI(5)P (23). PLIP demonstrated a $K_m$ of 5.9 mM.
against this substrate, more than 150-fold higher than the $K_m$ of 37.5 $\mu$m seen for di-C8 PI(5)P (Fig. 3).

Thus, PLIP demonstrated a clear preference for PI(5)P over any other proteinaceous or lipid substrate tested. Although our efforts to demonstrate the effect of PLIP on endogenous PI(5)P levels are ongoing, we note that in vitro observations have often served as indicators of in vivo function for PI phosphatases (10, 38–40).

**PLIP Is a Testis-enriched Phosphatase**—The relative tissue distribution of PLIP was determined by a Northern blot assay using anti-FLAG-agarose beads. Akt and phospho-Akt levels were probed via Western blot using anti-FLAG and anti-phosphoserine 473-Akt-specific antibodies, respectively. A third blot was performed on the cell lysates using anti-V5 (PLIP) to demonstrate the presence of wild-type and C132S PLIP. Note that because of a relatively higher level of Akt expression in the vector control cells, lanes 1 and 2 are derived from lower film exposure times. Results are representative of two separate experiments. C. Drosophila S2 cells were treated with buffer (U) or PLIP or PTEN dsRNA. Following treatment, endogenous Akt was immunoprecipitated and assayed for activity. The effectiveness of the dsRNA treatment, as assessed by mRNA levels, is shown in the blot below.

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**Fig. 5. Analysis of PLIP as an effector of Akt activity.** A. A proposed model of PI(5)P regulation of Akt activity, adapted from Carricaburu et al. (20). B. Wild-type (WT) PLIP, catalytically inactive (C132S) PLIP, or vector control were transfected along with FLAG-Akt into Chinese hamster ovary-insulin receptor cells and treated with (+) or without (−) insulin. Following immunoprecipitation with anti-FLAG-agarose beads, Akt and phospho-Akt levels were probed via Western blot using anti-FLAG and anti-phosphoserine 473-Akt-specific antibodies, respectively. A third blot was performed on the cell lysates using anti-V5 (PLIP) to demonstrate the presence of wild-type and C132S PLIP. Note that because of a relatively higher level of Akt expression in the vector control cells, lanes 1 and 2 are derived from lower film exposure times. Results are representative of two separate experiments. C. Drosophila S2 cells were treated with buffer (U) or PLIP or PTEN dsRNA. Following treatment, endogenous Akt was immunoprecipitated and assayed for activity. The effectiveness of the dsRNA treatment, as assessed by mRNA levels, is shown in the blot below.
The characterization of PLIP as a highly specific PI(5)P phosphatase is ongoing in our laboratory. We have shown that it possesses a distinct endogenous substrate not ascribed in vivo, the data weaken the idea that the lipid is capable of regulating Akt activity.

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9. The characterization of PLIP as a highly specific PI(5)P phosphatase is potentially important contribution to our understanding of PI regulation and adds a powerful tool for further analysis of PI signaling pathways within the cell. Until we achieve a clear demonstration of the ability of PLIP to alter PI(5)P levels in vivo, however, we cannot rule out the possibility that it possesses a distinct endogenous substrate not assessed in this study. Even if PLIP utilizes a highly specific proteinaceous substrate in vivo, the fact that it possesses this unique and well characterized preference for PI(5)P should make it possible to selectively measure PLIP activity under a variety of experimental conditions. Detailed investigations into the subcellular localization, in vivo enzymatic activity, and overall cellular function of PLIP are ongoing in our laboratory.
